Organ preservation and viability in kidney and liver transplantation

experimental and clinical studies

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Cover photo by M.H.J. Maathuis and C. Moers, courtesy of Mr James Gunn, York, UK, who took this picture in the winter of 2006 at the 'Festival of Angels' in York, UK.
The melting ice face symbolizes the main principle of current clinical organ preservation: metabolic suppression by hypothermia. Similar to the ever changing aspect of melting ice, the concepts in organ preservation are changing in order to improve outcome after organ transplantation.

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Scientiae enim naturalis non est simpliciter narrata accipere,
sed in rebus naturalibus inquirere causas.

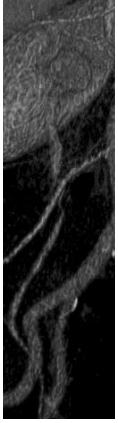
Experimentum solum certificat in talibus.

Albertus Magnus, 1262

Aan mijn ouders

Dankzij Jantien

CONTENTS



Chapter 1

Introduction
Perspectives in organ preservation

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Abstract

Maintaining organ viability after donation until transplantation is critically important for optimal graft function and survival. To date, static cold storage is the most widely used form of preservation in every day clinical practice. Although simple and effective, it is questionable whether this method is able to prevent deterioration of organ quality in the present era with increasing numbers of organs retrieved from older, marginal and donation after cardiac death donors. This chapter describes principles involved in effective preservation and focuses on the basic components and methods of abdominal organ preservation in clinical and experimental transplantation. Concepts and developments to reduce ischemia related injury are discussed including hypothermic machine perfusion. Despite the fact that hypothermic machine perfusion might be superior to static cold storage preservation, organs are still exposed to hypothermia induced damage. Therefore, recently some groups have pointed at the beneficial effects of normothermic machine perfusion as a new perspective in organ preservation and transplantation.

Introduction

Despite better insights in surgical technique, immunosuppressive agents and treatment of postoperative complications, five and ten year results in organ transplantation have only moderately improved in the past decades (1). One explanation for this slightly disappointing fact is that more experience has led to an increased acceptance of older and more complex recipient candidates. Another reason is the fact that due to the persistent shortage, criteria for inclusion of deceased donors have been extended. Organs are nowadays more often retrieved from older, more marginal and sometimes donation after cardiac death (DCD) donors than ten years ago. Between 1988 and 1995, the United Network of Organ Sharing (UNOS) registered a 170% increase in the number of deceased donors over 50 years of age (2;3). The use of older donor kidneys, livers and pancreata has resulted in a decrease in graft function and survival compared to grafts retrieved from young donors (4-7). Marginal and DCD donor organs suffer from additional warm ischemic injury. As a result these organs have higher primary non function (PNF) and delayed graft function (DGF) rates compared to heart-beating deceased donors (8-11).

Maintaining organ viability during preservation is an important prerequisite for successful outcome after transplantation. With the current practice to accept older and more injured donor organs, improvement of preservation techniques has now become a must. To date, most centers use static cold storage (CS) to preserve organs. This preservation method, however, was developed in an era with younger donors with good quality organs (12). With the introduction of extended donor criteria the limitations of CS have probably been reached.

This chapter aims to describe a number of principles and pathophysiological mechanisms as well as current techniques in abdominal organ preservation.

Organ preservation by static cold storage

Currently, CS is the preferred organ preservation method in most centers. Simple cold storage starts with a rapid vascular washout to allow cooling of the organ, removal of blood components and equilibrate the CS solution with the tissue (13;14).

Hypothermia

The principle of CS preservation is based on suppression of metabolism and catabolic enzymes by hypothermia (0-4°C). Metabolic rate is halved with each 10°C drop in temperature resulting in a remaining 10-12% metabolism at 4°C (15). Already in the early 1960s, it was shown that cooling by itself was able to improve preservation of small bowel, kidney and liver: The so called temperature effect (16-18). To further extend cold ischemic time (CIT) and counteract the detrimental side-effects of the required hypothermia, special preservation solutions are necessary: The solution effect (19). Cell swelling, acidosis and the production of radical oxygen species

Table 1. Composition of organ preservation solutions*

	EC (77)	HOC (15)	PBS (28)	UW (81)	HTK (87)	CEL (91)	IGL-1 (35)
Colloids							
HES, g/l	-	-	-	50	-	-	-
PEG-35, g/l	-	-	-	-	-	-	1
Impermeants (mM)							
Citrate	-	80	-	-	-	-	-
Glucose	195	-	-	-	-	-	-
Histidine	-	-	-	-	198	30	-
Lactobionate	-	-	-	100	-	80	100
Mannitol	-	185	-	-	38	60	-
Raffinose	-	-	-	30	-	-	30
Sucrose	-	-	140	-	-	-	-
Buffers (mM)							
Citrate	-	80	-	-	-	-	-
Histidine	-	-	-	-	198	30	-
K_2HPO_4	15	-	-	-	-	-	-
KH_2PO_4	43	-	-	25	-	-	25
$NaHCO_3$	10	-	-	-	-	-	-
NaH_2PO_4	-	-	13	-	-	-	-
Na_2HPO_4	-	-	56	-	-	-	-
Electrolytes (mM)							
Calcium	-	-	-	-	0.0015	0.25	0.5
Chloride	15	-	-	20	32	42	-
Magnesium	-	-	-	-	4	13	-
Magnesium sulphate	-	40	-	5	-	-	5
Potassium	115	79	-	120	9	15	25
Sodium	10	84	125	25	15	100	120
ROS Scavengers (mM)							
Allopurinol	-	-	-	1	-	-	1
Glutathione	-	-	-	3	-	3	3
Mannitol	-	185	-	-	38	60	-
Tryptophan	-	-	-	-	2	-	-
Additives (mM)							
Adenosine	-	-	-	5	-	-	5
Glutamic acid	-	-	-	-	-	20	-
Ketoglutarate	-	-	-	-	1	-	-

* Numbers between brackets represent references to relevant literature

EC: EuroCollins; HOC: Hypertonic Citrate / Marshall's solution; PBS: Phosphate-Buffered-Sucrose; UW: University of Wisconsin Cold Storage Solution; CEL: Celsior; HTK: Histidine-Tryptophan-Ketoglutarate; IGL-1: Institut Georges Lopez; HES: hydroxyethyl starch, PEG-35: polyethylene glycol with an average MW of 35 kDa, ROS: reactive-oxygen species.

(ROS) upon reperfusion are important side effects of hypothermia. To reduce these undesirable effects CS solutions include a number of specific compounds (20;21). The composition of several preservation solutions is illustrated in Table 1.

Cell swelling

A very prominent alteration in the cellular structure during hypothermia is the formation of edema (22). The responsible mechanism is an impaired activity of Na^+/K^+ ATPase. As a result, sodium is no longer extruded but passively enters the cell. This creates a hyperosmolar intracellular environment and subsequently an influx of water. To prevent cell swelling, impermeants and colloids are added to preservation solutions.

Effective impermeants are saccharides and non-saccharide anions. Molecular weight (MW) determines the effectiveness of saccharides to prevent cell swelling, with larger saccharides being more effective (23-25).

Glucose (MW 180) is a monosaccharide and was used in early CS solutions, e.g. EuroCollins solution. When it became evident that glucose passes the cell membrane and becomes a source of lactate in an anaerobic environment, it was no longer considered as an effective impermeant (26). The slightly larger monosaccharide mannitol (MW 182) is not a source of lactate since it is not metabolisable and will not enter the cell through facilitated transport. In addition, mannitol has a beneficial effect as a scavenger of reactive oxygen species, and was therefore added in Marshall's (hypertonic citrate; HOC), Bretschneider's Histidine-Tryptophan-Ketoglutarate (HTK) and Celsior (CEL) solution. Sucrose (MW 342) is a disaccharide and is used in the renal preservation solution Phosphate-Buffered-Sucrose (PBS) (27;28). Raffinose (MW 504) is the largest one and a trisaccharide. It was added as an impermeant in the University of Wisconsin CS solution (UW-CSS) developed by Belzer and Southard.

Non-saccharide impermeants such as negatively charged gluconate, citrate and lactobionate limit cell swelling by electrochemical forces. Effectiveness of these anions is determined by molecular weight as well as charge. While HOC contains citrate, both UW-CSS and Celsior use the anion lactobionate.

As impermeants are predominantly effective at the level of cell membranes and the interstitial compartment, colloids are used for the intravascular compartment. These macromolecules are retained in the vascular spaces and act by imparting colloid osmotic pressure. Colloids were originally added to hypothermic machine preservation solutions (MPS) to prevent tissue edema due to hydrostatic pressure. Belzer and his group first used cryoprecipitated plasma, than albumin and finally diafiltrated hydroxyethyl starch (HES) as they aimed at developing one solution suitable for both CS and hypothermic machine perfusion (HMP). The feasibility of HES as a colloid in UW-CSS has been extensively debated. HES prevents interstitial edema but also increases viscosity (29). For short preservation times addition of a colloid has been doubted, although some organs such as the pancreas appeared to be more susceptible to edema when HES is omitted (30). Analyzing the effect of HES on red blood cells (RBC), several authors have shown an increased RBC aggregability in both human and rat whole blood when large molecular sized HES

is present (31;32). This effect could partially explain the frequently slower washout of blood and initially patchy reperfusion of organs when UW-CSS is used in clinical practice (33).

The HES controversy initiated a search for other colloids, e.g. dextran and polyethylene-glycol (PEG) (34-36). In this respect, UW-PEG preserved rat livers have shown lower transaminase levels, higher bile flow and higher urea synthesis rate after transplantation (37). Several experimental studies have now confirmed the efficacy of PEG for liver as well as for kidney, pancreas and small bowel preservation (38-40).

In contrast to UW-CSS, both HTK and Celsior do not contain a colloid. In a prospective study with short CIT, both solutions showed equal efficacy compared to UW-CSS for the preservation of kidney and liver grafts (41;42). With prolongation of preservation times beyond 24 h, the presence of a colloid does appear to be important to maintain organ viability (43).

Energy and acidosis

At a temperature of 0-4°C cold storage results in a rapid depletion of cellular ATP. Within four hours nearly 95% of ATP has disappeared with a shift to AMP as the predominant nucleotide. During CS, anaerobic metabolisation of one mol glucose, however, only yields two mol ATP versus a maximum of 38 mol in aerobic glycolysis. Moreover, two lactic acid molecules are formed leading to acidosis (44;45).

The contribution of acidosis to ischemic injury is pH dependent. Severe acidosis activates phospholipases and proteases causing lysosomal damage and eventually cell death (46). Mild acidosis (pH 6.9-7.0), however, has been suggested to have a protective effect by inhibiting phosphofructokinase as the rate-limiting step in glycolysis (46;47). Adequate control of pH is therefore an important function of preservation solutions. UW-CSS and PBS use phosphate as a buffer, while Celsior and HTK use histidine. Of those two solutions, HTK has the highest buffering capacity due to a high concentration of histidine (21).

Reactive Oxygen Species (ROS)

ROS are widely recognized as important mediators of post-reperfusion induced organ injury (48). CS per se, however, has also been shown to promote ROS production, probably due to mitochondrial damage (49;50). An extensively studied generator of ROS is xanthine oxidase, which simultaneously produces hydrogen peroxide (H_2O_2) and the superoxide anion ($O_2^{\cdot-}$) (51;52). The subsequent reduction of H_2O_2, catalyzed by iron, leads to hydroxyl radical formation ($^{\cdot}OH$). Free or chelatable iron is not only a catalyst of ROS formation but also contributes directly to hypothermia induced injury by mediating mitochondrial damage and induction of apoptosis (53-55). ROS react rapidly with other molecules which will result in severe damage to lipids, nucleic acids and proteins (56;57). The subsequent cell death mechanism appears to be ATP dependent. ATP is required for the execution of the apoptotic cell death program whereas complete ATP depletion will lead to necrosis (58;59). As free radical mediated injury during preservation is strongly correlated with the absence of immediate and reduced long-term kidney function (57), preservation solutions aim

to counteract ROS mediated injury during preservation and especially at time of reperfusion.

In UW-CSS, the compounds allopurinol and glutathione (GSH) were included to prevent formation of ROS. Allopurinol inhibits xanthine oxidase, which improved kidney preservation, while liver or pancreas preservation remain almost unaffected (60).

GSH is a tri-peptide which is oxidized to glutathione disulphide together with converting peroxides. Experimental studies have shown the importance of GSH in an isolated perfused rabbit liver model. In the absence of GSH, more lactate dehydrogenase (LDH) was released into the perfusate (61) which was confirmed in the canine kidney transplant model. Subsequent studies have shown that GSH is especially important in long term liver preservation (62).

GSH is also used in Celsior solution, while in HTK tryptophan might protect the organs against ROS mediated damage. The anti-oxidative effects of tryptophan are, however, controversial. Tryptophan can act as an anti-oxidant through its oxidative metabolites in the kynurerine pathway, such as 5-hydroxy-tryptophan (63). On the other hand, tryptophan can be pro-oxidant as well by presenting low molecular weight iron in a redox cycling event (64;65). In a cultured rat hepatocyte experiment, the amount of thiobarbituric acid reactive substances (TBARS), as a marker for ROS mediated injury, was measured. After 24 h preservation, TBARS were significantly higher in HTK preserved hepatocytes compared to UW-CSS suggesting a superior anti-oxidant capacity of UW-CSS (66).

Electrolyte composition

During the pioneering years in organ preservation a high potassium/low sodium ratio of the solution (intracellular type) was assumed necessary to prevent cell swelling. It was hypothesized that due to the inactivity of Na^+/K^+ ATPase during hypothermia an intracellular sodium/potassium ratio in the extracellular fluid compartment would prevent sodium and chloride to enter the cell (67). Balancing extracellular sodium ions and intracellular protein anions creates the so called Donnan-equilibrium that prevents edema formation (24). Intracellular type solutions like UW-CSS were long considered to be pivotal for preservation of cell viability (68). Recent work, however, has suggested equal or improved results of extracellular type solutions with a low potassium/high sodium ratio, e.g. Celsior and HTK (69-74). This clearly demonstrates that sodium/potassium ratios as such do not play a central role in preservation. However, a low potassium content will probably facilitate the washout of blood during organ procurement as the potassium induced vasospasm is absent (71;75).

In summary: essential components of effective preservation solutions are impermeants or colloids, an adequate buffering capacity and anti-oxidants. In the next paragraph the clinical merits of some prominent preservation solutions for abdominal organs will be discussed.

Current cold storage solutions

The first static CS preservation solution was developed by G.M. Collins in 1969 (76), which was modified by the Eurotransplant Foundation in 1976 by eliminating magnesium (Table 1) (77). EuroCollins (EC) solution was a simple and cheap intracellular type preservation solution. Phosphate was used for pH buffering and glucose served as the osmotic agent. In the late seventies, an Australian group developed a hypertonic citrate (HOC) solution which is also known as Marshall's solution. This solution was effective for 72 h canine kidney preservation and is still in clinical use (78). Another simple solution is Phosphate-Buffered-Sucrose (PBS) developed by Coffey and Andrews in the early eighties. PBS was shown to be effective in kidney preservation confirming the hypothesis that high concentrations of impermeant saccharides suppress hypothermic cell swelling (27;79). When UW-CSS became available, a randomized clinical trial comparing EuroCollins with UW-CSS in kidney preservation showed that DGF was significantly lower in the UW-CSS group (23% vs. 33%). Also, one year graft survival was found to be significantly higher in the UW-CSS group (29). As a result of this study EC was no longer the preferred solution for clinical abdominal organ preservation in Europe (Fig. 1A).

University of Wisconsin Solution

Continuous and systematic research by Belzer and Southard led to the development of the University of Wisconsin Solution in 1987. Metabolic inert substrates such as lactobionate and raffinose served as osmotic agents. HES was used as a colloid. Scavengers (glutathione, allopurinol) and an ATP precursor (adenosine) were added to the solution. Today, UW-CSS is still considered the gold standard preservation solution for kidney, liver, pancreas and small bowel (Fig. 1). (29;80-86).

Histidine-Tryptophan-Ketoglutarate solution

Histidine-Tryptophan-Ketoglutarate solution (HTK) was initially introduced as a cardioplegic solution in open heart surgery by Bretschneider in the 1970s but was also tested in kidney, liver and pancreas transplantation (87). The basic design of the solution consists of histidine, a very potent buffer, combined with two (pre) amino acids. Tryptophan serves as membrane stabilizer while ketoglutarate acts as substrate for anaerobic metabolism during preservation. HTK has a low viscosity and to achieve complete tissue equilibration, high volumes (~15 l) have to be rinsed through the organs at low flow rates. A multicenter randomized clinical trial comparing UW-CSS versus HTK in kidney preservation showed equal results in terms of incidence of DGF (33% vs. 33%) (80). For prolonged cold storage times (> 24 h) little data is available. One single center study reported a two fold increase in incidence of DGF after HTK kidney preservation compared to UW-CSS when CIT was longer than 24 h (88). The opposite was shown in another study with a DGF rate of 16% after HTK preservation versus 56% after UW-CSS (89). Direct comparison of these conflicting findings, however, is impossible due to a different definition of DGF in both studies.

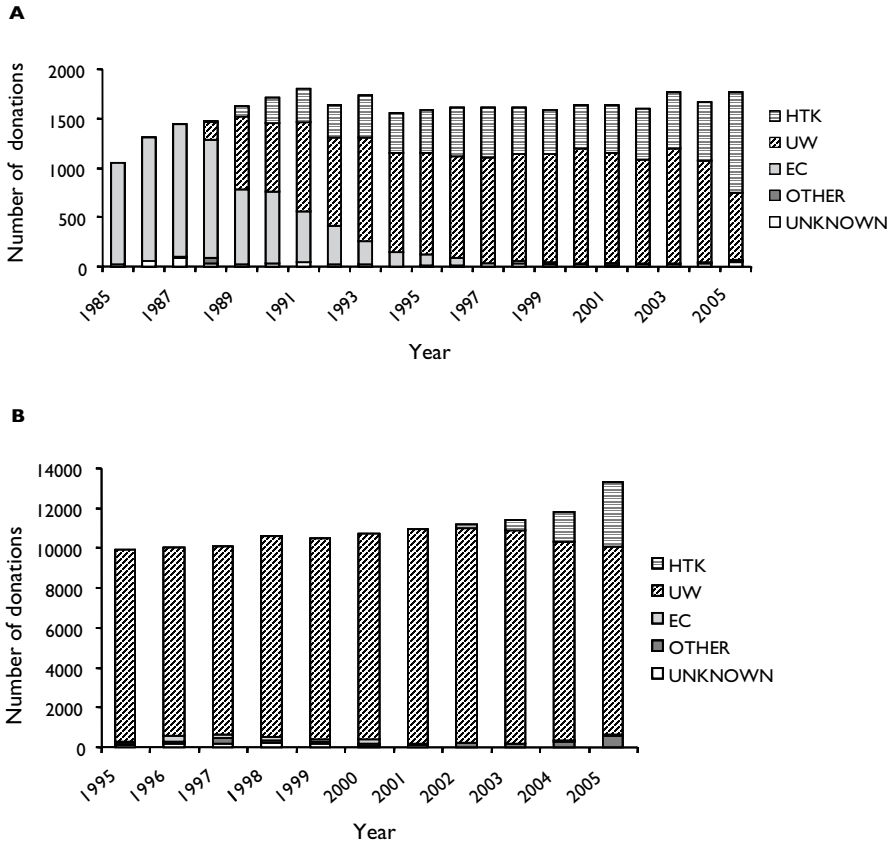

Figure 1. A. Use of cold storage solutions in Eurotransplant region in deceased donors from 1985-2005 (based on Eurotransplant data of October 2006).
B. Use of cold storage solution in the US in deceased donors from 1995-2005 (based on OPTN data of October 2006).

In liver preservation, it has been suggested that HTK could be advantageous due to its low potassium concentration. Therefore, the need to flush out the potassium-rich UW-CSS from the organ prior to reperfusion would be limited. Although patient numbers were relatively small and cold ischemic times short, two studies using HTK in liver preservation showed equality of HTK and UW-CSS for short term preservation (82;90). Despite the lack of a proper randomized and controlled trial, HTK is currently used by many centers as a preservation solution for all abdominal organs retrieved for transplantation (Fig. 1) (89).

Celsior

Celsior is an extracellular type preservation solution developed in 1994 for CS preservation of cardiac grafts (91). This solution, however, proved to be effective in preserving abdominal organs as well (41;84;85). It combines the inert osmotic agent philosophy of UW-CSS with the strong buffering capacity of HTK. Reduced glutathione is added as a free radical scavenger. Currently, it has been successfully used in clinical heart, lung, liver, pancreas, kidney and small bowel preservation (92;93).

New solutions

The increasing awareness that ischemia/reperfusion injury does determine a significant part of post-transplant outcome has stimulated research in the field of preservation injury and the development of new preservation solutions. A relatively new preservation solution developed at the University of Amsterdam is Polysol. Its composition is based on the fact that metabolism is still present at 4°C. Polysol has been tested both as an experimental CS solution and as HMP solution (94;95). It is a classic preservation solution enriched with amino acids, vitamins and anti-oxidants (96). Many components in Polysol, however, have not yet been evaluated separately. In experimental liver preservation studies, superiority over HTK was seen in CS preservation of steatotic livers showing improved functional parameters, e.g. oxygen consumption, bile production and damage markers (94). Transplantation data in experimental and clinical preservation are now required to demonstrate the efficacy of Polysol. Based on its 'metabolic support' design, however, beneficial effects of Polysol can be expected.

Another new and now clinically available preservation solution is IGL-1 (Institut Georges Lopez), developed by the Lyon group in France. IGL-1 builds on the heritage of both UW-CSS and Celsior (35;94;97). It combines the extracellular composition of Celsior with the colloidal support of UW-CSS using polyethylene glycol (PEG) instead of HES. In a porcine kidney autotransplantation model with IGL-1, PEG was found to limit influx of macrophages by approximately 50% (98). Polymers, such as PEG, spontaneously bind to cell and tissues surfaces and sterically stabilize the underlying surface from interactions with other components. The main advantage of this 'immunocamouflage', is that it directly modifies inherent immunogenicity of donor tissue (99;100). PEG does not exert any aggregating effects on RBCs and in combination with the extracellular composition of IGL-1, washout of blood during the donor operation should be superior to UW-CSS (31;101).

Both rat and porcine transplantation studies of liver and kidney have shown encouraging results in terms of organ function after transplantation following preservation with IGL-1 (42;70;102). The first preliminary clinical results in renal transplantation with IGL-1 demonstrated a reduction in DGF compared to kidneys preserved with UW-CSS (5.7% vs. 13.8%, respectively). Furthermore, less apoptosis was seen in IGL-1 preserved kidneys (103). Until now, however, patient numbers have been too small to draw clinically relevant conclusions and a randomized controlled multicenter study will

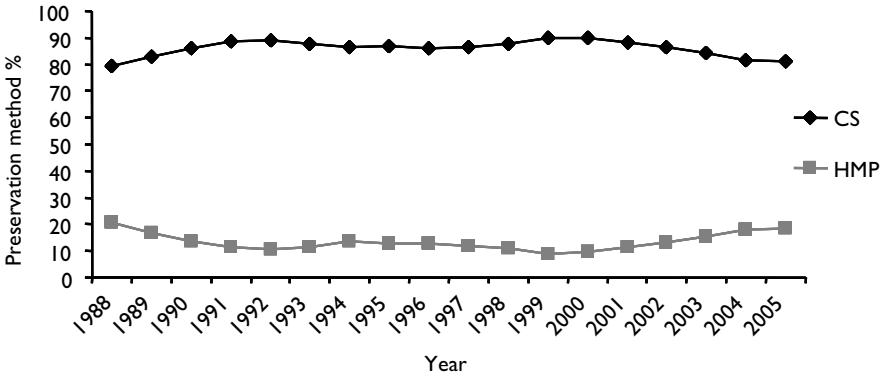

Figure 2. Relative amount of renal hypothermic machine preservation (HMP) and cold storage preservation (CS) in the US from 1988-2005. (based on OPTN data of October 2006).

have to confirm the initial results. Given its extracellular composition and the beneficial effects of PEG, IGL-1 could be considered a promising successor to UW-CSS.

Despite the fact that CS preservation methods have facilitated many transplant programs all over the world, it appears that the increasing challenge to maintain viability in extended-criteria donor organs is touching the limits of CS preservation. Even with beneficial additives and enriched compositions, static CS, at best, slows down ischemic damage. Furthermore, pre-transplant viability testing is limited and preservation time is still counted in hours rather than in days. To further improve organ viability, a more dynamic preservation method is needed to better fulfil the metabolic demands of damaged organs. Therefore, many groups have recently switched gears and are revisiting the possibilities of hypothermic machine perfusion (HMP) (104-106).

Preservation by hypothermic machine perfusion

In the early 1970s, hypothermic machine perfusion (HMP) was used by many centers in the United States and Europe to preserve kidneys, allowing transportation to a transplant center (107-110). Although modern HMP systems are smaller, lighter and more sophisticated than the original machine used by Belzer and co-workers, the principles of HMP have not changed.

Machine perfusion generates a controlled continuous or pulsatile recirculating flow of preservation solution at 0-4°C. This continuous flow allows complete perfusion of the organ promoting a thorough washout of blood and subsequent tissue

equilibration with the preservation solution. Beneficial effects claimed on behalf of machine perfusion are a low incidence of DGF, the possibility of real-time viability assessment, the ability to provide metabolic support during perfusion and the potential to add pharmacologic agents to the perfusate.

In kidney preservation, both in animal experiments and in historical controlled retrospective clinical studies, HMP has been demonstrated to provide better early graft function compared to CS (111;112). In addition, when kidneys retrieved from expanded-criteria, marginal or DCD donors were analyzed, HMP was found to be superior to CS (113-117). Unfortunately, in most studies no prospective randomization was performed and patient numbers were not large enough to allow extrapolation of the results. Recently, Wight and colleagues reported an excellent meta-analysis based on aggregated results of the current literature concerning HMP versus CS, clearly demonstrating a 20% reduction in DGF with HMP (118). DGF reflects a compilation of accumulated risk factors and depends on the presence or absence of independent donor, preservation and recipient characteristics (29). Possibly, some of the detrimental effects caused by these risk factors can be reduced with HMP. The occurrence of DGF requires continuation of dialysis and is associated with an increased incidence of acute rejection and inferior long-term outcome (119;120). While individual studies suggest potential benefits of HMP such as reduced DGF-rates, less acute rejection, and an improved better short and long-term function, no comparative study of these modalities has been performed under strict conditions (121;122).

Most experience with HMP concerns the kidney (Fig. 2). Only scarce experimental data exist in experimental liver transplantation by the groups of Belzer, Slapak and Brettschneider (123-125). Several strategies regarding perfusion of portal vein and/or hepatic artery have been applied. In 1986, D'Alessandro, and later Pienaar, from the Madison group, managed to successfully transplant canine livers after 72 h HMP (126;127). Clinical application of HMP in liver transplantation, however, has been limited to recent pioneering work of Guarrera and colleagues (104).

Overall, both experimental and clinical data suggest that HMP improves kidney and liver preservation. Modern, portable and stand-alone HMP systems for kidney preservation are now available, allowing user-friendly transportation within an international organ sharing system. Therefore, a broader clinical application of HMP should be considered to reduce the impressively high DGF rate of 60-85% in DCD kidneys and possibly reduce the Achilles heel in liver transplantation: Ischemic type biliary lesions (128-131).

New approaches in organ preservation

Apart from HMP, several other concepts have been developed to allow expansion of the donor pool. During the past decades, not only age but also the type of organ donors has changed. The cause of death has shifted from a relative healthy donor

with cerebral trauma to older patients suffering from cerebral hemorrhage. As a result, average donor organ quality has decreased and the task to at least maintain the quality of the graft before transplantation has become much more important.

A rather unusual but attractive technique to resuscitate damaged kidneys and livers is the perfusion of gas through the vasculature. This concept was initially described by Bunzl in 1954 and named "persufflation" by Isselhard in 1972. It consists of retrograde venous application of humidified pure oxygen (O_2) at 13-18 mmHg during CS (132;133). Renal persufflation preservation has been applied clinically in a small pilot study including ten paired kidneys. Although numbers were small, persufflated kidneys did show improved initial function compared to CS (134). Its application in liver preservation was extensively studied by the group of Minor. In several experiments they showed that gaseous oxygenation during CS was highly effective in improving liver graft viability (135-137). Using this method, survival after 45 min of warm ischemia in a DCD liver transplant model was 100%, compared to 0% in the CS group (137).

Another, more static way to deliver O_2 to CS grafts is the dual layer perfluorocarbon technique. Perfluorocarbons (PFC) are hydrocarbons in which most of the hydrogen atoms have been replaced with fluorine. The attractive property of PFC is a very high capacity for dissolving O_2. PFC liquids can store 20-25 times greater amounts of O_2 than water or blood. In addition, the very low O_2 binding constant of PFC allows a more effective release of O_2 in tissue than haemoglobin does. These properties make PFC-based solutions interesting for organ preservation (138).

PFC was first used in organ preservation as a component of the two-layer method (TLM) (139). The TLM is comprised of UW-CSS and oxygenated PFC for pancreas preservation. During preservation by TLM, canine pancreas grafts continuously generated ATP up to 96 h (140). In animal models, TLM appeared to be useful not only for pancreas but also for small bowel preservation (141). In the clinical setting, however, TLM remains controversial since it did not improve whole pancreas transplantation (142). Furthermore, there is debate about its effects on islet isolation. Although some small clinical trials have reported beneficial effects, the largest and most recent survey did not demonstrate superiority over UW-CSS in the field of human islet isolation (143-145).

The alternative approaches mentioned above, all have in common that they take advantage of the beneficial effect of O_2 during hypothermic preservation. Improving the energy status of organs during preservation leads to earlier recovery, especially in ischemically damaged organs. Whereas O_2 supports metabolism, various other gaseous compounds that act on signal transduction have also proven their efficacy to improve graft viability in the experimental setting. Donor pre-treatment with carbon monoxide (CO) at low concentrations in a rat small intestine transplant model reduced pro-inflammatory interleukins and improved survival to 100% compared to 58% in air-treated controls (146). Similarly, in rat liver transplants, exposure of the recipient to CO suppressed induction of TNF-α, iNOS and ICAM-1. Liver grafts showed improved liver function and less neutrophil infiltration after CO exposure (147). Nitric oxide (NO), the radical produced from L-arginine by the enzyme NO synthase (NOS), is

a potent vasodilatator that inhibits platelet and neutrophil aggregation and adhesion (148;149). This effect is potentially beneficial for preservation. Vasodilatation will improve organ washout during procurement, whereas the immunological effects of NO may limit reperfusion damage. Adding NO during cold ischemia improved small bowel viability in both rat and pig autotransplantation models (150). In addition, topical exposure of rat kidneys with NO significantly reduced the effects of 60 min warm ischemia (151).

These experiments suggest that exposure of the graft to CO and/or NO during preservation might induce a protective effect before reperfusion. HMP devices could enable administration of these compounds, either via an oxygenator as a gas or by pharmacological donors in the solution.

Outlook

As often before in transplant history, major improvements in preservation will probably be derived from new philosophies instead of adaptations of current strategies. Ideally, good preservation should facilitate the use of marginal and older organs and provide real-time viability assessment before transplantation. Normothermic (37°C) or sub-normothermic (25-32°C) machine perfusion (NMP) is becoming popular as a preservation alternative that may indeed achieve these goals (152). In canine kidney transplantation after 120 min warm ischemia, 18 h normothermic perfusion allowed eventual recovery of normal renal function, whereas primary non-function occurred in all kidneys preserved for 18 h with static CS (153). Raising the temperature during preservation provides more adequate ways to test and optimize graft viability and allows elimination of hypothermia induced injury (45;154).

Normothermic perfusion of the abdominal organs using a cardiopulmonary bypass system followed by CS has already been applied in human kidney transplantation. This so called normothermic recirculation protocol showed significant improvements in a group of 44 DCD kidneys. PNF and DGF rates were 0% and 12.5%, respectively, compared to 22.5% and 55% for conventional preservation techniques. Despite the fact that this study was retrospective and included patients over a 12 year period, it suggests a potential benefit for clinical application of normothermic techniques (155).

In liver preservation, normothermic perfusion of porcine livers subjected to 60 min of warm ischemia resulted in functioning liver grafts, whereas the animals transplanted with CS livers all died. Normothermic perfused livers demonstrated stable metabolic function with adequate production of coagulation factors, hyaluronic acid clearance, glucose metabolism and significantly lower transaminases compared to CS grafts (152;156;157).

The voluminous perfusion set-up, necessity of continuous monitoring during perfusion and technical complexity, however, have limited clinical application of

NMP so far (154;158). To introduce NMP as a feasible option in clinical practice a combination of techniques has to be used. After an initial period of conventional hypothermic preservation, allowing transportation to a specialized facility, NMP can be started. In kidney preservation it has been shown that HMP with intermittent NMP improves graft survival of canine kidneys after 30 min warm ischemia (159). The liver, however, is more vulnerable. Recently, initial NMP for 24 h was compared to 4 h CS followed by 20 h NMP in a porcine DCD model with 60 min of warm ischemia. The latter combination, however, was ineffective as the benefits of NMP were lost due to the short CS period (160).

Overall, NMP offers several advantages over conventional preservation techniques. Therefore, the development of a portable and easy-to-handle stand-alone device is crucial for the introduction of NMP into day-to-day practice for kidney and liver preservation.

Conclusion

Organ preservation has always been crucial for transplant outcome, but will become even more important in the present era with increasing numbers of older, more marginal and DCD donors. Although CS has proven its efficacy in the past, it seems that the limitations of this technique have been reached. To maintain organ viability, more efforts are necessary to reduce ischemia/reperfusion injury and initiate repair. Awaiting the results of several clinical trials, hypothermic machine perfusion or even normothermic machine perfusion may be (re)introduced in clinical preservation in general or for special categories of donor organs.

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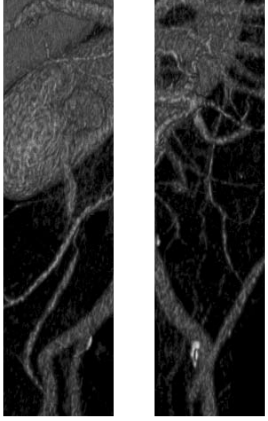

Chapter II

Rationale of the studies in this thesis

Static cold storage (CS) using the University of Wisconsin solution (UW-CSS), which was developed in the 1980s, is currently the most widely method for kidney and liver preservation. The success of kidney transplantation, however, is compromised by delayed graft function (DGF) rates as high as 20-30%, resulting in decreased graft survival and subsequent re-transplantation. In liver transplantation a primary non function (PNF) rate of 5-10% and an initial poor function (IPF) rate of 20-25% reduce transplantation effectiveness. When looking at donor demographics, the average deceased donor today is older, has suffered from a CVA rather than head trauma, and has more concomitant morbidity which negatively influences graft quality compared to donors in the 1980s. Furthermore, usage of organs derived from donation after cardiac death (DCD) is increasing and it is well known that these organs, which suffered from additional warm ischemia, have even higher DGF rates. Improved organ preservation might be able to reduce the high DGF, PNF and IPF rates and allow a successful expansion of the donor pool. The use of an improved organ preservation solution or a different preservation modality like hypothermic or normothermic machine preservation (HMP / NMP) may help to reach these goals. In *Chapter I* an overview is given on the main principles in organ preservation and the wide variety of preservation solutions and methods.

The recognition that organ preservation is an important factor in transplantation outcome has led to the development of new static cold storage preservation solutions. An example of such a new cold storage preservation solution is IGL-1, which was developed by the Edouard Herriot Hospital in Lyon, France. In contrast to UW-CSS, IGL-1 does not contain hydroxyethyl starch (HES), but instead polyethylene glycol (PEG) is used for colloidal support. HES induces red blood cell aggregation as was previously demonstrated in our laboratory (1). PEG does not cause aggregation of red blood cells (2). Another important advantage of PEG is its ability to sterically limit immune activation after reperfusion. Finally, IGL-1 has a low potassium content, preventing a potassium induced vasospasm and thereby improving wash-out efficacy. Based on these compositional differences the evaluation of this new preservation solution was started in the isolated perfused kidney (IPK) model. This model offers unique possibilities to study selected effects of ischemia/reperfusion injury under controlled conditions. In *Chapter III* the IPK model was used to focus on renal vasomotor function as a functional marker for vascular viability after wash-out and cold storage using either IGL-1 or UW-CSS.

The IPK model proved to be adequate for studying vasomotor functions but has some important limitations. Both the short period of approximately 90 minutes, in which adequate organ function is maintained and the use of an a-cellular perfusate limit proper assessment of post-preservation organ viability. Therefore in *Chapter IV* a rat kidney transplantation model was used to compare renal function and ischemia/reperfusion injury after static cold storage preservation using either IGL-1 or UW-CSS.

Although static cold storage is simple and effective it might not be sufficient anymore in the current era with an increasing portion of older, more marginal and DCD donors. This recognition has led to a renaissance of HMP. Although clinical kidney preservation in the 1960s started with HMP, this technique was subsequently abandoned on financial and practical grounds when CS was developed in 1969. For a successful re-introduction of HMP into an international organ sharing system like Eurotransplant, the system has to be portable, easy to use and stand-alone. Therefore, a new HMP system that fits these criteria was recently developed in our group (3). The Groningen Machine Perfusion (GMP) system was initially designed for liver preservation and makes use of blood compatible cardiopulmonary bypass machine components including an oxygenator. In *Chapter V* the results of a functional evaluation of the GMP system in a porcine liver preservation model are reported.

To be able to effectively study and compare organ viability after HMP using the GMP a large animal transplantation model is mandatory. Liver transplantation in the pig, however, is a very demanding, complex and expensive experimental model. By switching to a kidney transplantation model a proof of principle experiment using the GMP system could be performed. In *Chapter VI* the re-design of the GMP system for kidney preservation is presented including its evaluation in a porcine autotransplant model. In this study kidney preservation with HMP using the GMP system at a pressure of 30/20 mmHg or 60/40 mmHg was compared to CS using UW-CSS. Focussing on graft function, renal injury and endothelial damage the most optimal perfusion regimen for hypothermic machine perfusion of porcine kidneys was defined.

Athough static cold storage has been the preferred method of organ preservation for most transplant centers in the world, retrospective studies have suggested that HMP may result in improved short-term outcome, with lower DGF rates after kidney transplantation from all types of deceased donors (4;5). No data from sufficiently powered randomized controlled trials on the effect of HMP versus CS in clinical kidney transplantation are available at this point. Based on our experience with HMP in experimental kidney transplantation we started a prospective European multicenter study in co-operation with the Eurotransplant organization to determine the efficacy of HMP in clinical kidney transplantation. A commercially HMP device was used for this trial since the GMP system was not certified for clinical application. In *Chapter VII* the results of the first prospectively randomized controlled clinical trial on HMP versus CS in kidney transplantation are reported.

While HMP might be able to improve organ preservation, it is still based on the concept of hypothermia. Well known side effects of hypothermia include acidosis, cell swelling, and formation of radical oxygen species. Therefore, several research groups are currently exploring the possibilities of preserving organs at normothermic temperature (37°C) to avoid hypothermia induced damage (6-10). Since the metabolic rate of organs is significantly higher at these temperatures, traditional organ preservation solutions cannot be used. The extra demand for nutrients, oxygen and

buffer capacity cannot be fulfilled by solutions designed for traditional cold storage. The perfusion solution most frequently used in experimental normothermic preservation is diluted blood derived from the donor. In preparation of normothermic preservation using the GMP system and considering the sensitivity of endothelial cells in *Chapter VIII* the effects of circulating diluted blood on endothelial activation were studied in a porcine model.

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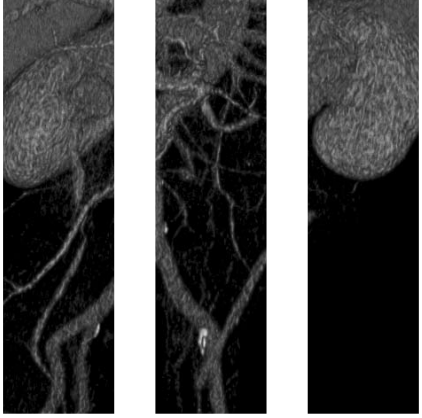

Chapter III

Deterioration of endothelial and smooth muscle cell function in DCD kidneys after static cold storage in IGL-1 or UW-CSS

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Abstract

Background: Kidneys obtained from donors after cardiac death are damaged by the combination of warm and cold ischemia. Although the parenchymal damage of these kidneys is well studied, little is known about the functional effects of warm and cold ischemia on the renal vascular bed. Kidney preservation using the new extracellular-type cold storage (CS) solution from Institut Georges Lopez (IGL-1) was compared with the University of Wisconsin solution (UW-CSS) with a special focus on vasomotor functions.

Methods: The influence of warm and cold ischemia on vasomotor functions was studied in an isolated perfused kidney (IPK) model. Six groups of DCD rat donor kidneys were studied with warm ischemia of 0, 15 and 30 minutes followed by 0 or 24 h cold storage (CS) preservation in IGL-1 or UW at 4°C. Endothelial dependent vasodilation was studied using acetylcholine, smooth muscle cell (SMC) constriction was assessed using phenylephrine (PE) and finally endothelial independent relaxation was tested using papaverine-sulphate.

Results: Smooth muscle cells were significantly affected by cold ischemia showing a 50% reduction of PE mediated constriction after preservation. Additional warm ischemia did not affect SMCs. After UW-CSS preservation endothelial dependent vasodilation was only significantly reduced when the combination of warm and cold ischemia was present. IGL-1 preserved kidneys showed a reduction in endothelial dependent vasodilation after isolated warm ischemia. Both preservation solutions rendered equal results after 24 h preservation.

Conclusion: Vasomotor functions are negatively influenced by the combination of warm and cold ischemia. Both IGL-1 and UW-CSS performed equally in preserving vasomotor functions. The interesting finding of the rapid decline of smooth muscle cell function might point at the first step towards intimal hyperplasia as seen in late transplant dysfunction.

Introduction

The main principle of static cold storage preservation is lowering of metabolism by cooling the organ (1). Although hypothermia is pivotal to organ preservation at this moment, it has negative side effects and induces extra injury to the organ. Both the vasculature and parenchyma of retrieved donor organs are affected by hypothermic preservation. In liver preservation ballooning of hepatocytes can be found after prolonged cold storage. In kidney preservation, the tubules are most affected by hypothermia (2;3). Focussing on the vasculature, two structurally and functionally different important components of the (arterial) vessel wall are the endothelium and the smooth muscle cells (SMCs). Endothelial cells are known to be very susceptible to ischemia/reperfusion injury (IRI) (4;5). Little is known, however, about the susceptibility of SMCs to IRI. Adequate preservation of organs with attention for the endothelium as well as for the SMCs is relevant for both short-term and long-term outcome of organ preservation. Rademacher et al. demonstrated the clinical relevance of vascular viability using Doppler ultrasonography in over 600 renal transplant patients. His group could show a significant correlation between the arterial resistance index measured after 3 months post transplant and long term graft function and graft survival (6). Focussing on long term failure, transplant arteriosclerosis (TA) is the primary cause of allograft loss after the first year post transplant. A common histomorphological feauture of TA is a concentric proliferation of SMCs, endothelial swelling and myocyte necrosis. The etiology of TA is multifactorial but donor related risk factors are thought to be important in the development of TA (7). Especially organs donated after cardiac death (DCD), where a combination of warm and cold ischemia is present, are expected to suffer extra damage in comparison to heart beating donation (8). Most studies addressing the problem of vascular damage after preservation, focus on structural alterations or use isolated aortic artery ring segments which are mounted in a myograph to study endothelial cell vasomotor function (5;9;10). There is, however, evidence that aortic endothelial cells are different from renal artery endothelial cells in terms of functional and morphological changes after transplantation (11). In an allogeneic rat model both kidney and aorta were transplanted. In the aorta allograft vasoconstriction was absent within two weeks after transplantation, whereas in the main renal artery this response was fully maintained up until 33 weeks posttransplant. This experiment elegantly demonstrates that the renal artery appears to be more resistant to transplantation in comparison to an aorta allograft. In kidney transplantation, however, not only the renal artery but the entire renal vascular bed is transplanted. Therefore, to study the effects of IRI on vascular functioning it is necessary to use a method that enables the assesment of the entire vacular bed, instead of merely the renal artery. The isolated perfused kidney model (IPK) with continuous pressure monitoring is an adequate model to assess endothelial and SMC reactions to various stimuli before and after preservation (12). In this study the effects of warm and cold ischemia on renal vasomotor function were investigated using two different preservation solutions. The first preservation solution is the University of Wisconsin solution (UW-CSS), which is currently the most widely used kidney preservation solution (1). The second

Table 1. Composition of IGL-1 and UW cold storage solution

Component	UW-CSS Concentration	IGL-1 Concentration
Lactobionate	100 mM	100 mM
KH_2PO_4	25 mM	25 mM
$MgSO_4$	5 mM	25 mM
Raffinose	30 mM	30 mM
Allopurinol	1 mM	1 Mm
Gluthathione	3 mM	3 mM
Adenosine	5 mM	5 mM
Hydroxy-ethyl starch	0.25 mM	0
Macrogol (PEG) 35	0	0.03 mM
Na^+	25 mM	120 mM
K^+	120 mM	25 mM
pH	7.4	7.4
Osmolality	320 mOsm/L	320 mOsm/L

preservation solution is Institut Georges Lopez-1 (IGL-1), which was developed by the Lyon Group in 1997 (13-18). With regards to the present study a few aspects of these preservation solutions are important. The sodium and potassium content of UW-CSS is based on the intracellular sodium/potassium ratio in human cells with a relative high potassium content. In contrast to UW-CSS, IGL-1 has an extracellular composition and contains polyethylene glycol (PEG) instead of hydroxyethyl starch (HES, Table 1). PEG is a non-toxic synthetic colloid that reduces lipid peroxidation and has immune-modulating properties and, unlike HES, PEG does not cause red blood cell (RBC) aggregation (14;16;19-22). Based on these properties IGL-1 might be better capable of preserving vasomotor function.

The goal of this study is twofold. The first goal is to study the extent of vascular injury, with a focus on smooth muscle and endothelial cell vasomotor function. The second objective is to assess whether IGL-1 is capable of ameliorating the effects of warm and cold ischemia. This study is performed in a DCD animal model with various warm ischemic periods followed by 24 h CS with either UW-CSS or IGL-1.

Animals, materials and methods

Animals

Male inbred Fisher rats (F344), weighing 250-300 gram, were used as organ donors (Harlan, Horst, The Netherlands). All experimental procedures were approved by the Animal Experiments Committee of the University of Groningen. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed.

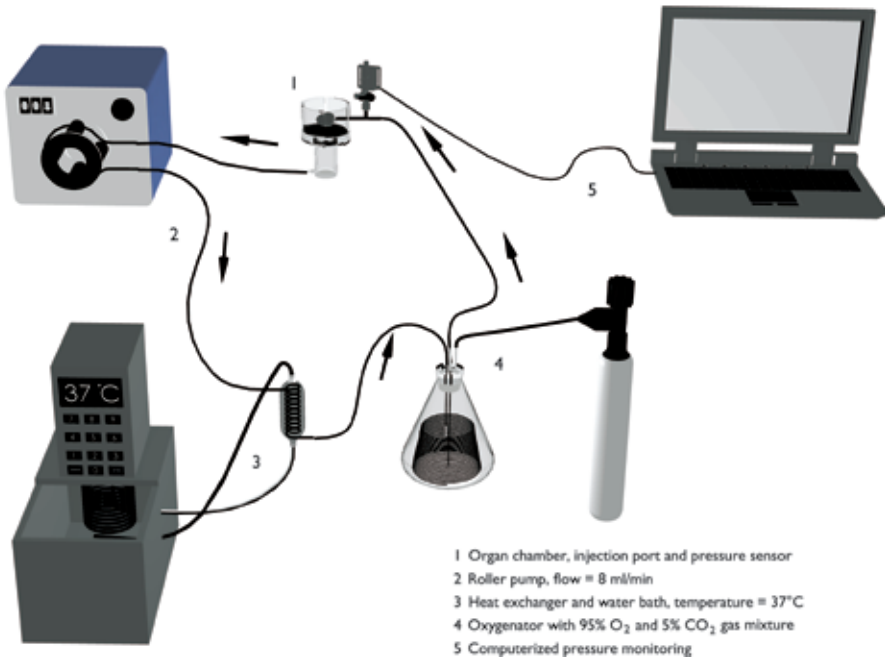

Figure 1. Schematic drawing of the setup of the IPK-perfusion system.

Experimental groups

To study the effects of warm and cold ischemia on vasomotor function six groups (n=6) with increasing periods of warm ischemia time (WIT), with and without static cold storage (CS), were studied.

- Group 1: 0 min WIT, 0 or 24 h CS using UW-CSS (control group for UW-CSS);
- Group 2: 0 min WIT, 0 or 24 h CS using IGL-1 (control group for IGL-1);
- Group 3: 15 min WIT, 0 or 24 h CS using UW-CSS;
- Group 4: 15 min WIT, 0 or 24 h CS using IGL-1;
- Group 5: 30 min WIT, 0 or 24 h CS using UW-CSS;
- Group 6: 30 min WIT, 0 or 24 h CS using IGL-1.

Experimental design

After a WIT of 0, 15 or 30 minutes (min) one kidney was randomized to direct assessment of vasomotor function in the isolated perfused kidney model (IPK, Fig. 1). Both endothelial dependent vasodilation using acetylcholine (ACH) and endothelial independent vasoconstriction using phenylephrine (PE) were assessed (Fig. 2). These measurements provided baseline data on the influence of WIT on vasomotor function. The other kidney was preserved using 24 h CS with UW-CSS or IGL-1. Following preservation, the combined effect of WIT and cold storage on vasomotor function was subsequently investigated in the IPK.

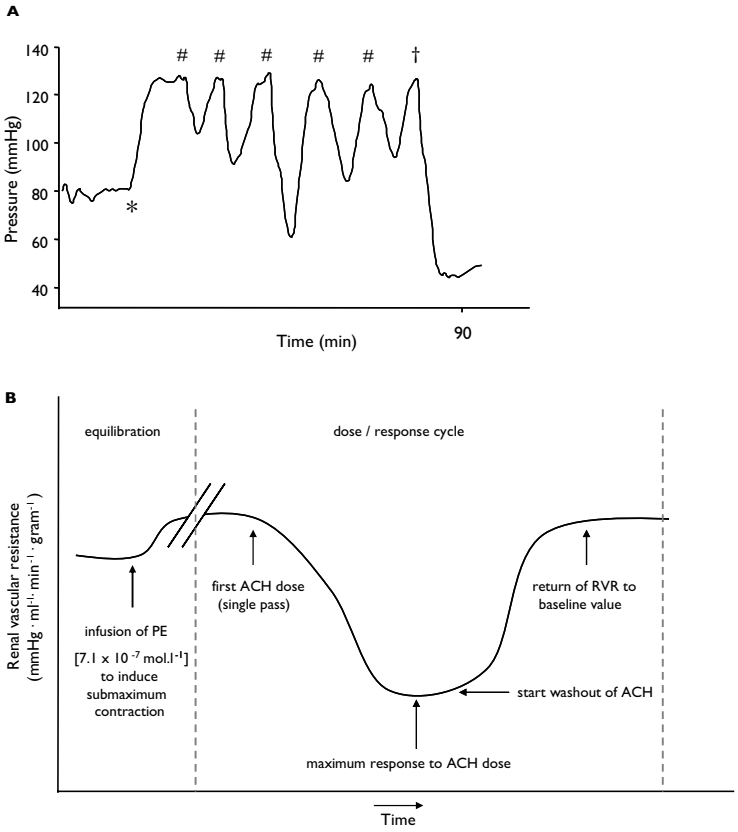

Figure 2. **A.** Typical example of recorded intra renal pressure changes during the course of one experiment. Pressure readings were used in calculation of renal vascular resistance changes (Fig. 2B). *Phenylephrine (PE) infusion resulting in an increase of pressure. # bolus injections of acetylcholine (ACH) using increasing doses (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} mol.l^{-1}) resulting in a dose dependent decrease in pressure. † Bolus injection of papaverine-sulphate resulting in a decrease in pressure.
B. Schematic representation of changes of renal vascular resistance (RVR) during one dose/ response cycle. After equilibration of RVR, phenylephrine (PE) infusion was started to obtain submaximum contraction. When RVR had stabilized, the first dose of acetylcholine (ACH) was given. After maximum ACH response and subsequent return to baseline RVR the next ACH dose was given.

DCD donor procedure

After induction of isoflurane anaesthesia, donors were heparinized with 250 IU heparin through the penile vein. Both kidneys and renal vessels were isolated via a midline incision. After opening the thoracic cage, cardiac arrest was induced by manual cardiac tamponade for 5 minutes (23). Following cardiac arrest a warm ischemic period of 0, 15 or 30 minutes was applied. This period resembles the clinical situation of a DCD donor. After this period, a ligature was placed superior to

the renal arteries to prevent flushing of liver and intestine. The kidneys were routinely flushed with 5 ml of 0.9% NaCl at 37°C immediately followed by 5 ml IGL-1 or UW-CSS solution at 4°C. The composition of these solutions is described in Table 1.

Preservation

During CS, kidneys were submerged in 25 ml of preservation solution at 0-4°C during 24 h in a 50 ml falcon tube (Greiner, Bio-One B.V., Alphen aan den Rijn, The Netherlands).

Reperfusion model and endothelial function

In the isolated perfused kidney (IPK) model (Fig. 1), the kidney was continuously perfused via the renal artery with 700 ml warmed (37°C) and oxygenated (95% O_2 and 5% CO_2 gas mixture) Krebs-Henseleit-Bicarbonate (KHB) solution at a pH of 7.4 ± 0.05 and a pO_2 > 100 kPa, by using a roller pump (Ismatec mv-ca/04, Ismatec, Glattbrugg, Switzerland) delivering a constant flow (8 ml/min) throughout the experiment (24). The composition of the KHB was as follows (mmol.l^{-1}). NaCl: 118; KCL: 4.7; $CaCl_2$: 2.5; KH_2PO_4: 1.2; $MgSO_4$: 1.2; $NaHCO_3$: 25; glucose: 10. After connecting the kidney, the flow was gradually increased to 8 ml/min and the first 50 ml of perfusate were discarded to avoid contamination with preservation solution. Drugs were either constantly perfused or given as bolus injections into an injection port 2 cm from the renal artery. Vascular responses were monitored by an electromechanical pressure transducer (Cobe, Arvada, USA) connected to a computer program (LabVIEW, National Instruments, Austin, USA). Changes in renal vascular resistances were subsequently calculated and adjusted for kidney weight. After an equilibration period of 25-30 min, when renal vascular resistance (RVR) had stabilized, perfusion with PE at a concentration that causes submaximum constriction (7.1 x 10^{-7} mol.l^{-1}, Sigma-Aldrich, Steinbach, Germany) was initiated and continued until the end of the experiment. After the PE-induced vasoconstriction had reached a plateau, subsequent doses (1.10^{-5} mol.l^{-1} to 1.10^{-1} mol.l^{-1}) of 0.1 ml acetylcholine (ACH, Sigma-Aldrich) were given single pass by bolus injection (Figs. 2A and 2B). The next dose of ACH was not given until RVR had returned to baseline. Time between doses varied between 1.5-2.5 min. ACH causes endothelial dependent vasodilation and was used to investigate the functional effects of warm ischemia and CS on endothelial cells. Vascular responses to every ACH-dose were quantified by the maximum decrease in RVR. After the last ACH dose of 1.10^{-1} mol.l^{-1}, 0.1 ml of 0.5% papaverine-sulphate (Centrafarm, Ettenleur, The Netherlands) was given to induce endothelial independent SMC relaxation and demonstrate SMC viability.

Statistics

Data are expressed as mean ± standard error of mean (SEM). Statistical significance of differences between groups was assessed by the Mann-Whitney U test. A p-value ≤0.05 was considered to indicate statistical significance.

Results

Baseline renal vascular resistance (RVR) in control kidneys was 7.8 ± 0.6 mmHg·ml^{-1}·min^{-1}·g^{-1}. No differences between the two tested solutions were observed. Similar values (data not shown) were obtained in groups with 15 and 30 minutes warm ischemic kidneys. Preservation during 24 h using either solution did not result in a significant increase of RVR.

Smooth muscle cells

The addition of PE caused constriction of the SMCs as illustrated by a rapid increase of the RVR varying from 6.1 to 8.8 mmHg·ml^{-1}·min^{-1}.g^{-1}, in control kidneys and kidneys that were subjected to warm ischemia only. The response to PE, however, was signficantly reduced with 50-68% in kidneys that were preserved for 24 h in either solution. This suggests a hypothermia induced loss of SMC vasoconstriction function (Fig. 3).

When studying endothelial independent vasodilation, the capacity of SMCs to relax using papaverine-sulphate showed a reduction of RVR of 5.1± 0.8 mmHg·ml^{-1}·min^{-1}·g^{-1} in control kidneys. Similar values (data not shown) were obtained in warm ischemic and preserved kidneys. No differences were observed between IGL-1 and UW-CSS. This data proofs that SMCs are capable of endothelial independent vasodilation after warm and cold ischemia.

Endothelial cells

To study endothelial cell dependent vasodilation bolus injections of ACH were given and dose response curves could be made, showing the relation between ACH dose and reduction in RVR (Fig. 4). In kidneys not subjected to warm ischemia, CS did not affect ACH induced vasodilation, showing similar reductions in RVR before and after 24 h CS preservation in either solution. In UW-CSS, the combination of warm ischemia and CS caused a significant decrease in the effectiveness of ACH to induce vasodilation, suggesting a loss of endothelial cell function. In IGL-1 a reduction in ACH induced vasodilation was already present after isolated warm ischemia was present. In contrast to IGL-1, UW-CSS solution was able to preserve endothelial vasomotor function after 15 and 30 min of warm ischemia without additional cold ischemia. Following 24 h CS, however, there was no difference between the two solutions in terms of endothelial cell function.

Discussion

Successful organ preservation should result in adequate recovery of all components of the transplanted organ including the vasculature. In this study we compared the cold storage preservation solution IGL-1 to UW-CSS and focused on vasomotor functions.

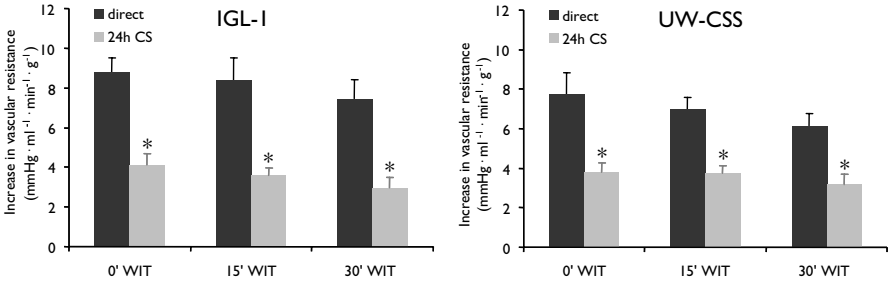

Figure 3. Phenylephrine responses in IGL-1 groups (left) and UW-CSS groups (right) before and after 24 h preservation following 0, 15, or 30 min of warm ischemia (WIT). Irrespective of warm ischemia the phenylephrine response was reduced after 24 h preservation. No differences between solutions were observed. * $p<0.05$ vs. direct.

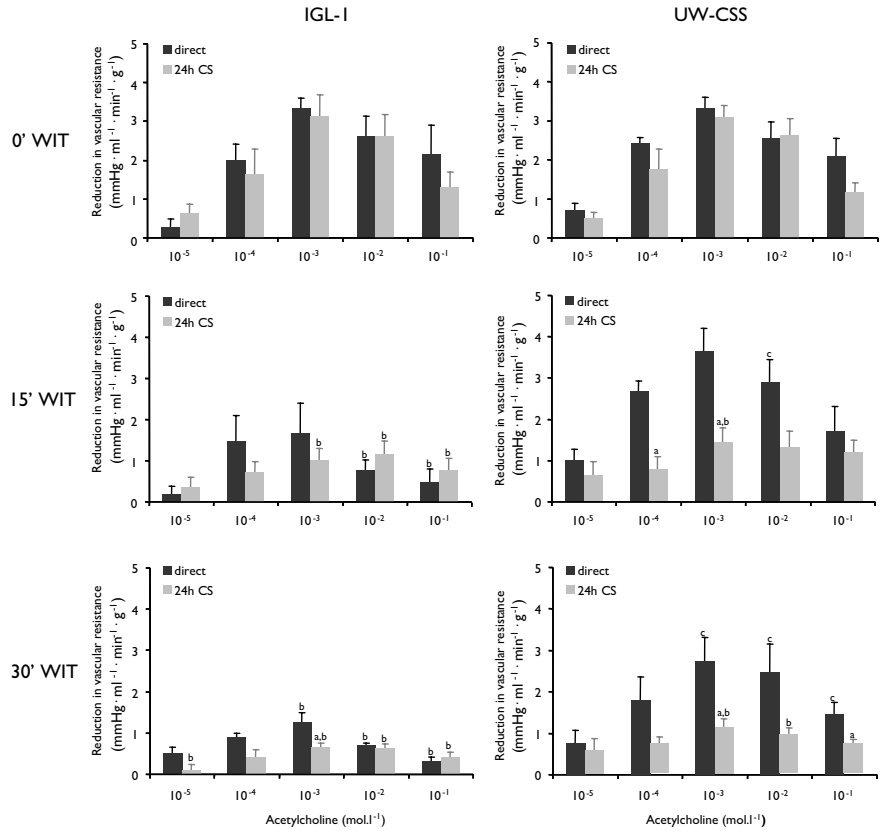

Figure 4. Acetylcholine dose response curves on rat renal vascular beds after 0, 15, or 30 min warm ischemia (WIT) before and after 24 h preservation using IGL-1 (left) or UW-CSS (right). After 24 h preservation, no differences between solutions were observed.
a: $p<0.05$ vs. direct, b: $p<0.05$ vs. control, c: $p<0.05$ vs. other solution.

Using the isolated perfused kidney model (IPK) we could investigate the effects of warm ischemia and cold storage on vasomotor functions of the entire renal vacular bed. By using different vasoactive substances we could study endothelial cell and SMC function.

The endothelium is a key player in mediating normal vascular phsyiology. Exposure to hypothermia during static cold storage alters the phsyiological and biochemical characteristics dynamics of endothelial cells (25). Morphologic studies of cold-stored blood vessels show cytoplasma vacuolisation and cell swelling of endothelial cells after prolonged storage times (10). Several *in-vitro* studies on coronary and aortic segments have shown a negative effect of cold storage on vasomotor functions (5;26;27). Less is known, however, about whole organ endothelial function (12).

Our results show that the combination of warm and cold ischemia results in a reduction of endothelial dependent vasodilation. We suggest that this is caused by a reduction in NO production and NO transport due to a loss of aquaporins (AQPs) in endothelial cells.

Acetylcholine induced endothelial cell relaxation was not affected by cold storage in the absence of warm ischemia. Both IGL-1 and UW-CSS were equally capable of preserving endothelial dependent vasodilation after 24 h CS if no warm ischemia was present. Vasodilation in reaction to acetylcholine was, however, significantly reduced in kidneys subjected to both warm ischemia and static cold storage. In contrast to this reduced responsiveness to acetylcholine, the reaction to papaverine-sulphate, was not affected. The positive reaction to this NO independent and endothelial independent vasodilator proves that the SMC apparatus was capable of vasodilation. This suggests that combined warm and cold ischemia, found clinically in the DCD setting, affect either NO production by endothelial cells or the reaction to NO by SMCs. Recently, it has been shown that aquaporin-1 (AQP-1) is a very important NO transporter, transporting NO out of endothelial cells and into vascular SMCs. AQP-1 is a transmembrane protein that is present in water transporting cells such as the proximal tubule cell but also in endothelial cells and SMCs, in which water transport is not a key function. In AQP-1 -/- knockout mice NO-dependent relaxation of thoracic aortas was found to be impaired (28). Another study showed that AQPs are sensitive to warm ischemia, resulting in decreased expression and functioning of AQPs following a warm ischemic insult (29). A possible explanation of our findings in the present study is that warm ischemia might have reduced the expression of AQPs and thereby impaired NO transport from the endothelial cell to the SMC. More research is needed to study this hypothesis.

Looking at the difference in potassium content of IGL-1 and UW-CSS the present experiment shows that potassium concentration is not an important factor in renal endothelial cell preservation. Concerns have been raised regarding potential endothelial injury from the high potassium concentration in UW-CSS. Several studies in the field of cardiac preservation have stated that high potassium concentrations damage the endothelium dependent vasodilatory response (30;31). In contrast, one study reported superiority of high potassium containing solutions like UW-CSS and EuroCollins over a low potassium solution like HTK in endothelial preservation (32). In

the present study both IGL-1, with a low potassium content, and UW-CSS, with a high potassium content, resulted in equal 24 h preservation of renal vascular endothelial response to acetylcholine, showing that potassium is not pivotal in renal endothelial cell preservation. Furthermore, the direct reaction to ACH in warm ischemic kidneys was better when kidneys were flushed-out using UW-CSS. Although this UW-CSS benefit does not easiliy translate to the clinical situation, where a (short) period of CS is always present, it shows that flush-out using high potassium content solutions does not harm renal endothelial function.

A novel finding of this study is that smooth mucle cells are more susceptible to cold storage than endothelial cells. In contrast to endothelial cell vasomotor function, both IGL-1 and UW-CSS could not prevent a reduction in SMC constriction after PE stimulation following cold storage. SMCs, the effectors of vasomotor responses, are generally considered to be sufficiently well resistant to hypothermia(33). The present study demonstrates, however, that SMCs are injured by 24 h CS with and without warm ischemia. A marked reduction of approximately 50% in contractile response to phenylephrine was seen after 24 h CS, which was, in contrast to endothelial cells, not further affected by additional warm ischemia. Presumably, cold ischemia is detrimental for SMC function in contrast to warm ischemia. These data show that rat renal vascular SMCs are less tolerant to hypothermia than previously thought. Looking at transplant arteriosclerosis, which is a late cause of graft failure characterized by a concentric proliferation of SMCs, we hypothesize that damage to the SMCs during cold storage contributes to this late event. This 'early hit' hypothesis is supported by experimental kidney transplant models which show a correlation between prolonged preservation time and the development of transplant arteriosclerosis (7;34;35).

In conclusion, we compared IGL-1 and UW-CSS in their efficacy to preserve renal vascular motor functions after warm and cold ischemia. The combination of warm and cold ischemia negatively affects endothelial dependent vasodilation. SMCs seem to be more tolerant than endothelial cells when it comes to warm ischemia but more suspectible for hypothermia induced damage. Both solutions were equally capable of preserving vasomotor functions after 24 h preservation and subsequent assessment in the isolated perfused kidney model.

Acknowledgments

The authors gratefully acknowledge the valuable technical assistance of Petra Ottens and Renee Gras during the experiments. Furthermore we would like to thank Arjan van der Plaats for his drawing of the IPK model and IGL (Le Pontet, France) for providing preservation solutions and financial support for this study.

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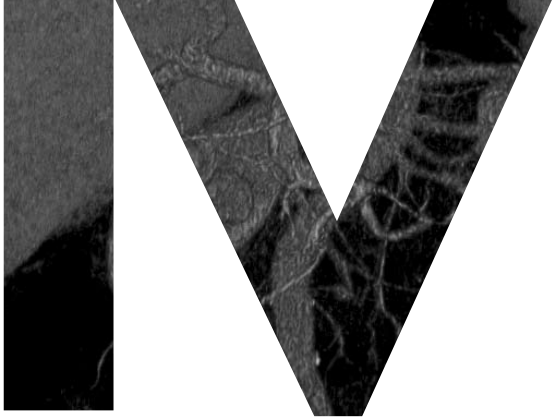

Chapter IV

Static cold storage preservation of ischemically damaged kidneys. A comparison between IGL-1 and UW-CSS

MHJ Maathuis, PJ Ottens, H van Goor, JJ Zwaagstra, J Wiersema-Buist, TA Schuurs, RJ Ploeg and HGD Leuvenink

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Abstract

Background: Nowadays kidneys derived from donation after cardiac death (DCD) are used in many centers to reduce donor shortage. Especially in these damaged organs, adequate organ preservation is critically important to maintain graft viability. IGL-1 is a new preservation solution, with an extracellular sodium/potassium ratio and polyethylene glycol (PEG) as a colloid.

Methods: The influence of warm and cold ischemia on renal damage and graft function was evaluated in a Lewis-Lewis rat transplant model with a follow up of 14 days. Eight groups of donor kidneys were studied with warm ischemia of 0 and 15 minutes followed by 0 or 24 h cold storage preservation in IGL-1 or UW-CSS. Blood was collected daily during the first week and at day 14. Recipients were placed in metabolic cages at days 4 and 14 after transplantation allowing urine collection and adequate measurement of glomerular filtration rate.

Results: Focussing on inflammation, reactive oxygen species production, proximal tubule damage, proteinuria, histology, and renal function after transplantation we could not show any relevant difference between IGL-1 and UW-CSS. Furthermore, the combination of 15 minutes warm ischemia and 24 h cold ischemia did not result in life sustaining kidney function after transplantation, irrespective of the used solution.

Conclusion: In the present experiment, static cold storage preservation of ischemically damaged rat kidneys in either IGL-1 or UW-CSS led to equal results after transplantation.

Introduction

To date, static cold storage (CS) using University of Wisconsin solution (UW-CSS) is the most frequently used kidney preservation method. It consists of a rapid vascular wash-out allowing cooling of the organ, removal of blood and equilibration between CS solution and tissue (1). The increasing awareness, however, that ischemia/reperfusion injury does significantly affect outcome after transplantation has stimulated research of preservation damage and the development of new preservation solutions (2). A new and now clinically available preservation solution is Institut Georges Lopez-1 (IGL-1), developed by the Lyon group in 1997 (3-8). In contrast to UW-CSS, IGL-1 has an extracellular composition and contains polyethylene glycol (PEG) instead of hydroxyethyl starch (HES). PEG is a synthetic colloid that reduces lipid peroxidation, has immune-modulating properties and does not cause red blood cell (RBC) aggregation (4;6;9-12). Recommended wash-out volumes are comparable with UW-CSS while the pricing of IGL-1 is approximately 50% of UW-CSS. Both, the extracellular composition and the use of PEG in IGL-1 could be an important advantage over UW-CSS, especially in the donation after cardiac death (DCD) setting (13).

The aim of these transplant experiments was to compare the efficacy of IGL-1 in preserving ischemically damaged rat kidneys to UW-CSS. In a DCD rat transplant model, kidneys were explanted after 0 or 15 minutes warm ischemia followed by 0 or 24 h static cold storage preservation in IGl-1 or UW-CSS. After preservation, kidneys were transplanted in an isogeneic recipient directly followed by a bilateral native nephrectomy.

Animals, materials and methods

Animals

Inbred male Lewis rats, weighing 250-300 g, obtained from Harlan (Zeist, The Netherlands), where used as kidney donors and recipients. All experimental procedures were approved by the Animal Experiments Committee of the University of Groningen. All animals showed normal renal function before the start of the study. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed.

Experimental design

The animal model used was a modification of an established rat kidney transplantation model including bilateral native nephrectomy (14). Warm ischemia times (WIT) of 0 min and 15 min were studied. Cold ischemia times (CIT) were either 0 h, i.e. only wash-out, or 24 h. Donor kidneys (n=7 per group) were recovered immediately or after 15 min following cardiac arrest and subsequently preserved for 24 h using either IGL-1 or UW-CSS. Eight experimental groups were studied:

- Group 1: 0 min WIT and 0 h CS with IGL-1;
- Group 2: 0 min WIT and 0 h CS with UW-CSS;

- Group 3: 15 min WIT and 0 h CS with IGL-1;
- Group 4: 15 min WIT and 0 h CS with UW-CSS;
- Group 5: 0 min WIT and 24 h CS with IGL-1;
- Group 6: 0 min WIT and 24 h CS with UW-CSS;
- Group 7: 15 min WIT and 24 h CS with IGL-1;
- Group 8: 15 min WIT and 24 h CS with UW-CSS.

DCD donor procedure

After induction of isoflurane anaesthesia, donors were heparinized with 250 IU heparin through the penile vein. The left kidney, renal vessels and ureter were isolated via a midline incision. After opening the thoracic cage, cardiac arrest was induced by manual cardiac tamponade for 5 min (15). A ligature was placed superior to the left renal artery to prevent flushing of the right kidney, liver and intestine. The explanted left kidney was routinely flushed with 2.5 ml of 0.9% NaCl at 37°C immediately followed by 2.5 ml IGL-1 or UW-CSS solution at 4°C by means of a 20 G needle inserted at the aortic bifurcation. The left kidney was removed including patches of aorta and caval vein.

Preservation

Kidneys in groups 5-8 were submerged in 25 ml of preservation solution at 0-4°C during 24 h in a falcon tube (Greiner, Bio-One B.V., Alphen aan den Rijn, The Netherlands). Kidneys in groups 1-4 were immediately transplanted with a cold ischemic time ranging from 23-28 min.

Recipient procedure

To allow stress-free and undisturbed blood sampling, recipient animals received a permanent jugular vein cannula with subcutaneous tunnelling of the cannula to a head attachment device one week prior to transplantation (16). After complete recovery, demonstrated by a return to pre-cannulation bodyweight, animals were eligible for transplantation. Time between jugular vein cannulation and transplantation did not exceed seven days. The microsurgical transplantation technique used in this study was a modification of the technique described by Lee (17). Prior to reperfusion, the graft was flushed with 1 ml of 0.9% NaCl at 4°C to wash out preservation solution. The renal vessels were anastomosed end-to-side to the recipient's aorta and caval vein using 9-0 prolene suture (Johnson & Johnson, Brussels, Belgium) and the ureter was anastomosed end-to-end to the recipient's ureter using 10-0 prolene sutures (Johnson & Johnson, Brussels, Belgium). The vascular clamps were released immediately after the vascular anastomosis was completed, with a mean warm ischemia time ranging from 20 to 25 min. At this point both native kidneys were removed.

Blood collection

Blood samples (0.4 ml) were taken daily until day 7 and on day 14 after transplantation. Fluid replacement was obtained using 0.4 ml 0.9% NaCl. Subsequently, 0.08 ml 65% poly vinyl pyrrolidone solution with 5000 IE heparin/ml was inserted in the

catheter's lumen to prevent clotting. After centrifugation, the plasma was collected and stored at -80°C until further analysis.

Urine collection

On days 4 and 14 after transplantation rats were housed for 24 h in individual metabolic cages, with access to drinking water. Urine volume was determined gravimetrically and samples were subsequently stored at -80°C until further analysis.

Tissue preparation and biochemical analysis

Sacrifice

At day 14 rats were anesthetized with isoflurane followed by cannulation of the aorta and a 5 ml blood sample was subsequently taken. The kidney graft was perfused in situ with 10 ml 0.9% NaCl at 4°C to obtain optimal tissue morphology. After removal of the kidney, it was divided in three pieces. Both upper and lower poles were snap frozen in liquid nitrogen. The middle pole was halved and stored in 4% formalin or embedded (Tissue-Tek, Zoeterwoude, The Netherlands) and snap frozen in liquid nitrogen.

Microscopic techniques

Tissue was collected, fixated in 4% formalin, subsequently paraffin embedded and cut into 3 µm thick sections. Light microscopy (20x magnification) of haematoxylin and eosin (HE) and periodic acid-Schiff (PAS) stained sections were used to demonstrate changes in morphology.

Immunohistochemistry

To assess the extent of tubular and interstitial injury in the different groups, immunostaining for α-smooth muscle cell actin (pre-fibrotic changes; α-SMA, clone 1A4, Sigma St. Louis, MO, USA), osteopontin (marker of tubular injury; OPN, clone MPIIIB10, Developmental Hybridoma Studies, Iowa City, IA, USA) and macrophages (ED-1, Serotec, Oxford, UK) was performed. As previously described, deparaffinized sections (3 µm) were subjected to heat-induced antigen retrieval by overnight incubation in 0.1 M Tris.HCl buffer (pH 9.0) at 80°C (18). Endogenous peroxidase was blocked for 30 min with 0.3% H_2O_2 in PBS. Primary antibodies (Table 1), diluted in 1% BSA/PBS, were incubated for 60 min at room temperature. Binding was detected using sequential incubations (30 min, Table 1) with appropriate peroxidase-labeled secondary antibodies (DakoCytomation) diluted in PBS with 1% BSA and 1% normal rat serum. Peroxidase activity was determined using 3,3'-diaminobenzidine tetrachloride (DAB) for 10 min. Relevant sections were counterstained with haematoxylin.

Measurements in blood and urine

Plasma creatinine levels were determined using a routine clinical laboratory test (CREA plus, Roche) which has been validated for measurements of rat serum creatinine concentrations (19). Urinary creatine levels were determined by the Jaffé method (Sigma-Aldrich Chemie b.v., Zwijndrecht, The Netherlands). ThioBarbituric

Table 1. Antibodies used for immunohistochemistry and primers for RT-PCR

Specificity	Primary Antibody	Supplier	Dilution Primary Antibody	Secondary / Tertiary Antibodies
α-SMA	Monoclonal mouse anti-SMA clone 1A4	Sigma St. Louis, USA	1:10000	Rampo/Garpo 1:100
Osteopontine	Monoclonal clone MPIIIB10	Developmental Hybridoma Studies, Iowa City, USA	1:250	Rampo: 1:50 Garpo: 1:100
ED-1	Monoclonal mouse anti-ED-1	Serotec, Oxford, UK	1:750	Rampo/Garpo 1:100

Primer	Forward	Reverse	Amplicon (bp)
IL-18	5'-CAACCGCAGTAATACGGAGCATA-3'	5'-CAGGCGGGTTTCTTTTGTCA-3	62
ß-actine	5'-GGAAATCGTGCGTGACATTAAA-3'	5'-GCGGCAGTGGCCATCTC-3'	75
α-SMA	5'-GAGAAAATGACCCAGATTATGTTTGA-3'	5'-GGACAGCACAGCCTGAATAGC-3'	74
GAPDH	5'-GTATGACTCTACCCACGGCAAGTT-3'	5'-GATGGGTTTCCCGTTGATGA-3'	79
E-Selectin	5'-GTCTGCGATGCTGCCTACTTG-3'	5'-CTGCCACAGAAAGTGCCACTAC-3'	73
Collagen III	5'-AGTTCTAGAGGATGGCTGCACTAAAC-3'	5'-TCTCATGGCCTTGCGTGTT-3'	81
ICAM-1	5'-CCAGACCCTGGAGATGGAGAA-3'	5'-AAGCGTCGTTTGTGATCCTCC-3'	251
TNF- α	5'-AGGCTGTCGCTACATCACTGAA-3'	5'-TGACCCGTAGGGCGATTACA-3'	67

Rampo, peroxidase-conjugated rabbit anti-mouse antibody; Garpo, peroxidase-conjugated goat anti-rabbit antibody.

acid reactive substances (TBARS) in urine were analyzed as an indication of increase in lipid peroxidation by reactive oxygen species (ROS) after reperfusion (20). Malondialdehyde binds to thiobarbituric acid and the subsequently formed TBARS were extracted in a butanol layer, measured with a fluorescence spectrophotometer at 485/590 nm (Beun de Ronde FL 600, Abcoude, The Netherlands). Urinary protein content was assayed via the method of Lowry and colleagues (21). Activity of brush border enzymes alanine aminopeptidase (AAP) and lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) in urine were measured using colorimetric assays (22). NAG was measured using a modified enzyme assay according to Findlay (23) at pH 4.25 using *p*-nitrophenyl-n-acetyl-β-D-glucosaminide as a substrate. AAP was detected with the modified enzymatic assay of Pfleiderer (24) using alanine-*p*-nitroanilide as a substrate.

Measurement of renal damage

Focal glomerular sclerosis (FGS) was semiquantitively scored (scale 0-400) in PAS-stained sections and expressed as the mean score of 50 glomeruli/kidney. FGS was scored positive when mesangial matrix expansion and adhesion of the visceral epithelium to Bowman's capsule were simultaneously present. A score of 1 was given when 25% of the glomerulus was involved, 2 for 50%, 3 for 75% and 4 for 100% (25). Interstitial fibrosis (IF) was scored similarly in 30 interstitial fields. A score of 0 was given when no interstitial fibrosis was present in a field, 1 for 0-25%, 2 for 25-50%, 3 for 50-75% and 4 for 75-100% of the field showing IF. IF was defined as expansion of the interstitial space, with or without the presence of atrophied and dilated tubules and thickened tubular basement membranes (26). Computerized morphometry was used to quantify immunohistochemical data (18).

RNA isolation and quantitative PCR for inflammation, injury and fibrosis.

Real-Time quantitative RT-PCR analysis of interleukin-18 (IL-18), Tumor Necrosis Factor-alfa (TNFα), E-selectin, Intracellular Adhesion Molecule-1 (ICAM-1), Collagen-type III and α-SMA gene expression was performed to detect inflammation, endothelial damage, tubular injury or fibrosis 14 days after transplantation. Amplification primers (Table 1) were designed with Primer Express software (Applied Biosystems) and validated in a 6-step 2-fold dilution series. RNA was extracted from snap frozen tissue using TRIzol (Invitrogen, Breda, The Netherlands). Total RNA was treated with DNase I, Amp Grade (Invitrogen, Breda, The Netherlands). cDNA synthesis was performed from 1 µg total RNA using T_{11}VN oligo's and M-MLV Reverse Transcriptase, according to supplier's protocol (Invitrogen). Amplification and detection were performed with the ABI Prism 7900-HT Sequence Detection System (Applied Biosystems, Foster City, USA) using emission from SYBR green (SYBR Green master mix, Applied biosystems). All assays were performed in triplicate. After an initial activation step at 50°C for 2 min and a hot start at 95°C for 10 min, PCR cycles consisted of 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Gene expression was normalized with the mean of β-actin mRNA content and calculated relative to controls. Results were finally expressed as $2^{-\Delta CT}$ (CT threshold cycle).

Statistics

Data are expressed as mean ± standard error of mean (SEM). Area under the creatinine curve was determined using SigmaPlot software (Systat Software, Point Richmond, U.S.A.). Statistical significance of differences between groups was assessed by Kruskall-Wallis followed by the Mann-Whitney U test. For all analyses, a p-value ≤ 0.05 was considered to indicate statistical significance.

Table 2. Clinical parameters and damage markers

WIT (min) - CIT (h) Preservation solution	Group 1 0-0 IGL-1	Group 2 0-0 UW-CSS	Group 3 15-0 IGL-1	Group 4 15-0 UW-CSS	Group 5 0-24 IGL-1	Group 6 0-24 UW-CSS	Group 7 15-24 IGL-1	Group 8 15-24 UW-CSS
Anastomosis time (minutes mean ± SEM)	28 ± 1	30 ± 2	30 ± 1	27 ± 1	30 ± 2	26 ± 1	26 ± 1	25 ± 0
Peak serum creatinine (µmol/l mean ± SEM)	101± 28	105 ± 21	138 ± 32	130 ± 32	269 ± 77[a]	218 ± 23[a]	522± 17[a,b,c]	489± 24[a,b,c]
GFR day 4 (ml/min mean ± SEM)	2.1 ± 0.2	1.8 ± 0.3	1.9 ± 0.2	1.8 ± 0.3	1.5 ± 0.4	1.1± 0.2	N.A.	N.A.
GFR day 14 (ml/min mean ± SEM)	2.3 ± 0.2	2.3 ± 0.4	2.7 ± 0.3	2.6 ± 0.5	3.1 ± 0.5[d]	3.7 ± 0.6[d]	N.A.	N.A
Urine volume day 4 (ml/24 h mean ± SEM)	14 ± 1	19 ± 3	16 ± 4	19 ± 2	24 ± 6[a]	31 ± 4[a]	N.A	N.A.
Urine volume day 14 (ml/24 h mean ± SEM)	13 ± 1	17 ± 1a	13 ± 2	14 ± 2	23 ± 4[a]	26 ± 3[a]	N.A.	N.A.
Proteinuria day 4 (mg/l/24 h mean ± SEM)	79.2 ± 3.4	68.9 ± 4.2	85 ± 13.9	96.9 ± 18.9	205.1 ± 41.2[a,b]	132.6 ± 23.7[a]	N.A.	N.A.
Proteinuria day 14 (mg/l/24 h mean ± SEM)	152.0 ± 3.5[d]	168.3 ± 4.2[d]	149.7 ± 11.9[d]	178.0 ± 36.3	251.7 ± 44.8[a]	256.1 ± 16.6[d]	N.A.	N.A.
Urinary AAP day 4 (U/l mean ± SEM)	2.3 ± 0.7	1.8 ± 0.9	5.4 ± 1.7	0.4 ± 0.4[b]	5.9 ± 2.4	1.9 ± 1.4	N.A.	N.A.
Urinary AAP day 14 (U/l mean ± SEM)	6.1 ± 0.9[d]	6.4 ± 1.8[d]	5.4 ± 0.6	7.5 ± 1.5[d]	15.4 ± 7.6	8.6 ± 4.2	N.A.	N.A.
Urinary NAG day 4 (U/l mean± SEM)	0.33 ± 0.02	0.32 ± 0.04	0.49 ± 0.06[a]	0.42 ± 0.05	0.64 ± 0.07[a]	0.56 ± 0.04[a]	N.A.	N.A.
Urinary NAG day 14 (U/l mean± SEM)	0.42 ± 0.09	0.32 ± 0.04	0.35 ± 0.05	0.39 ± 0.04	0.44 ± 0.07	0.58 ± 0.07[a]	N.A.	N.A.
Urinary TBARS day 4 (µM / 24 h)	90.1 ± 6.4	78.6 ± 5.8	90.6 ± 9.2	107.3 ± 22.2	92.5 ± 13.4	111.9 ± 34.5	N.A.	N.A.

a: $p<0.05$ vs. 0-0; b: $p<0.05$ vs. 15-0; c: $p<0.05$ vs. 0-24; d: $p<0.05$ vs. day 4

Results

One microsurgeon performed all experimental procedures. No significant differences in anastomosis times were observed between groups (Table 2).

Survival

After a challenge of 15 min WIT in combination with 24 h CIT (15-24 groups), independent of the used solution all animals died within three days post-transplant. Death was caused by renal failure demonstrated by increasing serum creatinine levels (Fig. 1). Therefore, on animal welfare grounds, no further attempts were made to complete this group and inclusion was stopped after n=4 for both UW-CSS and IGL-1 groups. Survival in all other groups was 100%.

Renal function

Post-transplant renal function data are presented in Figure 1 and Table 2. Kidneys subjected to both 15 min WIT and 24 h CIT (15-24 groups) were severely damaged resulting in an irreversible rise of serum creatinine for both IGL-1 and UW-CSS kidneys. Serum creatinine levels at day 1 and day 2 and area under the creatinine curve were significantly higher ($p<0.05$) in kidneys subjected to 24 h CIT (0-24 groups) compared to control kidneys (0-0 groups). Renal dysfunction in this group was further demonstrated by significant ($p<0.05$) higher urine volumes at day 4 and 14 post transplantation compared to controls (Table 2). There was no difference between IGL-1 and UW-CSS. Serum creatinine levels of kidneys subjected to 15 min WIT (15-0 groups) were numerically higher compared to control kidneys but this did not reach statistical significance.

Glomerular filtration rate (GFR) was calculated using 24 h urine volume and urinary and serum creatinine levels (Table 2). On day 4 there was no significant difference between the GFR although a trend was seen towards a lower GFR in the 0-24 groups. After 14 days GFR was higher compared to day 4 for all groups but only statistically significant for the 0-24 group. No difference between the two solutions in renal function was observed.

Proximal tubule damage

Detection of NAG and AAP activity in urine allowed an assessment of injury to the proximal tubule at 4 and 14 days after transplantation (Table 2). Urinary concentrations of AAP were significantly higher in IGL-1 preserved kidneys at day 4 compared to UW-CSS. NAG levels measured at day 4 were significantly increased in 15-0 and 0-24 groups, compared to controls. No difference in NAG levels could be demonstrated between IGL-1 and UW-CSS.

ROS formation

Measurement of TBARS in urine at day 4 allowed detection of ROS production (Table 2). No difference in TBARS levels were demonstrated between IGL-1 and UW-CSS.

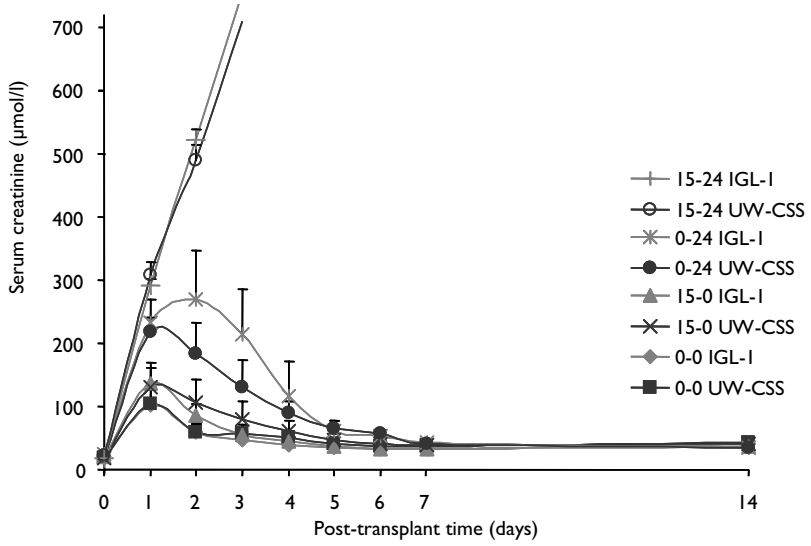

Figure 1. Post-transplant serum creatinine concentration in DCD rats after preservation with IGL-1 or UW-CSS.

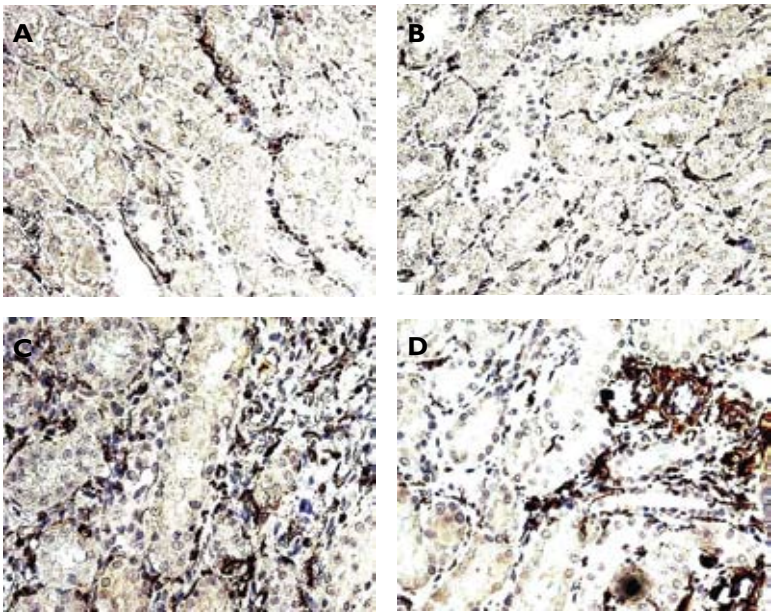

Figure 2. Representative section of kidney specimens after two weeks immunostained for ED-1 in 0-0 IGL **(A),** 0-0 UW-CSS **(B)**, 0-24 IGL **(C)** and 0-24 UW-CSS **(D)** preserved grafts. Significant more ED-1 staining was observed in both 0-24 groups compared to both 0-0 groups ($p<0.05$). No difference between IGL-1 and UW-CSS could be detected.

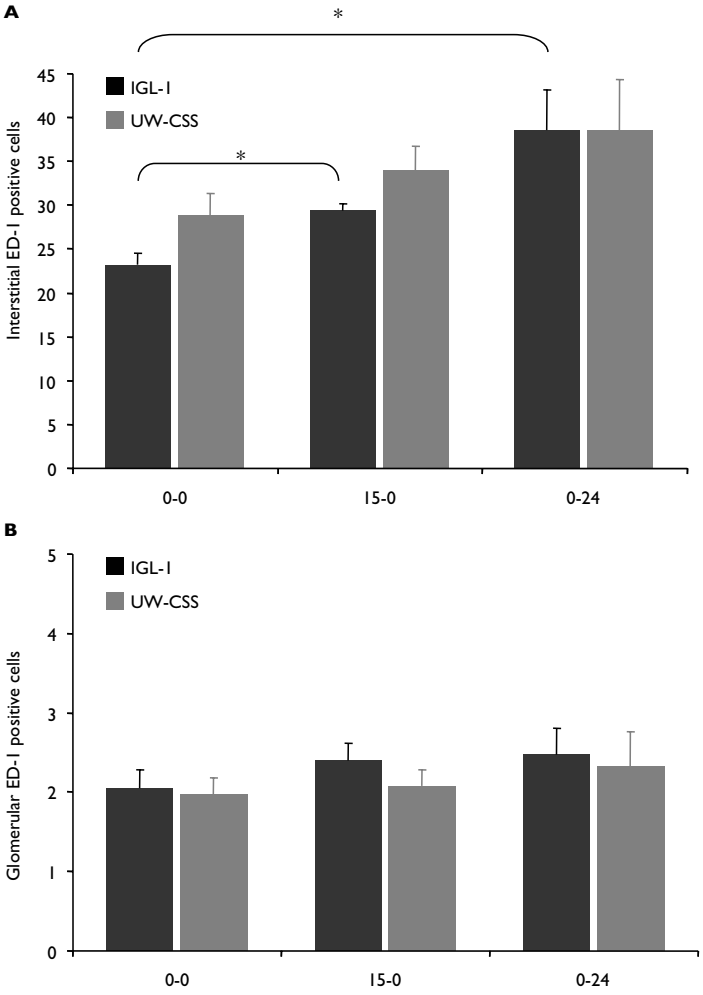

Figure 3. ED-1 positive cells in interstitium **(A)** and glomeruli **(B)** 14 days after transplantation using IGL-1or UW-CSS. * $p<0.05$.

Proteinuria

Urinary protein content was measured on day 4 and 14 after transplantation. On day 4, there was no difference between 15-0 groups and controls. 0-24 groups showed, however, a two fold significant ($p<0.05$) increase in urinary protein concentrations compared to controls (Table 2). At day 14, proteinuria was more profound in all groups compared to day 4 measurements. Again, 0-24 groups demonstrated the most severe proteinuria. No difference in proteinuria between IGL-1 and UW-CSS was seen.

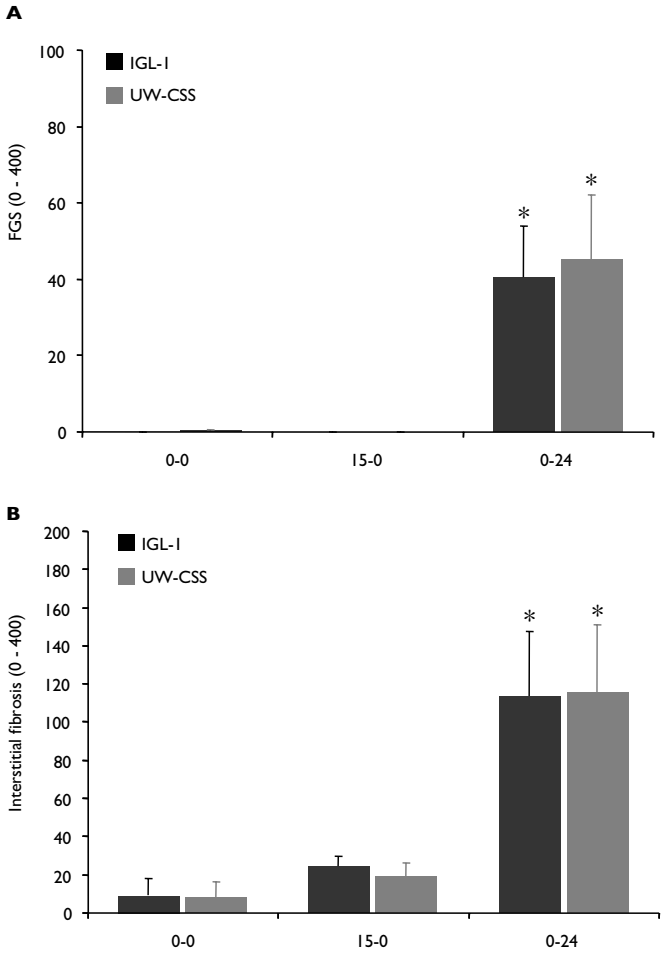

Figure 4. Focal gomerulosclerosis **(A)** and interstitial fibrosis **(B)** given in arbitrary units 14 days after transplantation using IGL-1or UW-CSS. * $p<0.05$ vs. other groups.

Immunohistochemical assessment of infiltrating cells

To detect any differences in immunoprotection of both solutions infiltrating ED-1-positive macrophages were identified by immunohistochemical analysis 14 days after transplantation (Fig. 2). A distinction was made between interstitial and glomerular infiltration. Interstitial analysis showed significant differences between groups in the number of ED-1-positive cells with more infiltration in 15-0 and 0-24 groups compared to controls (Fig. 3A). Glomerular infiltration was similar in all groups (Fig. 3B). In both glomerular and interstitial infiltration of macrophages no difference could be demonstrated between IGL-1 and UW-CSS.

Renal damage

There was a significant increase in FGS and IF in both 0-24 groups compared to controls. Both IGL-1 and UW-CSS preservation resulted in the same amounts of FGS and IF (Fig. 4A and Fig. 4B)

Real-Time quantitative Taqman RT-PCR analysis of IL-18, TNFα, E-selectin, ICAM-1 and α-SMA gene expression did not differ between groups. Computerized morphometry showed the same pattern.

Discussion

In the present study, a Lewis-Lewis rat kidney transplant model with and without warm and cold ischemia was used to compare the efficacy of IGL-1 and UW-CSS in terms of functional recovery from ischemia/reperfusion injury. As recipient animals were bilaterally nephrectomized at time of transplantation, this model is able to selectively study preservation effects of IGL-1 and UW-CSS. The present study shows equivalence of the new IGL-1 solution compared to UW-CSS in rat kidney transplantation.

Several ex-vivo experiments that have focused on static cold storage preservation of rat kidneys with (protoypes of) IGL-1 reported increased viability versus UW-CSS using an isolated perfused kidney (IPK) model for functional assessment (3;27). In the present study, however, we were not able to demonstrate any significant advantage of IGL-1 over UW-CSS. Although the IPK model offers an opportunity to control and manipulate renal function and has been well established in transplantation and pharmacology research, it has important shortcomings. Most relevant in the context of preservation experiments is the absence of whole blood reperfusion. In a transplant model blood reperfusion aggrevates the hypothermic induced structural damage (2;28). We therefore speculate that the extra damage in this transplantation experiment nullified the potential beneficial effects of IGL-1 as demonstrated previously in IPK models.

This experiment demonstrated that kidney function is negatively affected by 15 min WIT or 24 h CIT. A combination of both 15 minutes WIT and 24 h CIT did not result in life sustaining post-transplant kidney function. After four consecutive failures, inclusion in these groups was stopped. In the literature only a few studies combine both WIT and 24 h CIT in a rat model with direct bilateral nephrectomy. A study from Yin and colleagues, however, combining both 20 min WIT and 24 h static cold storage using UW-CSS confirms our results with 0% survival in a Lewis rat model (29). For the remaining groups the first GFR measurements was performed three days after transplantation allowing full postoperative recovery before putting the animals in metabolic cages. Although the area under the creatinine curve was significantly larger in the 0-24 groups compared to controls, GFR measurements at day 4 did not show a significant difference. Judging from the serum creatinine values, however, kidney function had already recovered by that time. Overall we were not able to demonstrate any functional differences between IGL-1 and UW-CSS.

Both warm and cold ischemia have drastic and duration-dependent effects on proximal renal tubule cells. Maintaining their integrity is crucial for early graft function as proximal tubule cells play a critical role in electrolyte, water and solute reabsorption from the glomerular filtrate. Therefore, the preservation efficacy of both IGL-1 and UW-CSS was compared with a special focus on the proximal tubule (30-32). Measuring both urinary AAP and NAG levels as proximal tubule specific damage markers we could demonstrate that both 15 minutes WIT and 24 h CIT result in significant proximal tubule injury compared to controls. Although AAP levels were significantly higher in IGL-1 compared to UW-CSS preserved kidneys after 15 minutes WIT, this was not the case with NAG levels. AAP reflects damage to the brushborder of the proximal tubule while urinary NAG is pointing towards intracellular, lysosomal damage. We therefore speculate that temporal differences in reconstitution of the brushborder rather than structural differences have caused this marked difference between IGL-1 and UW-CSS. The extent of proteinuria at day 4 and 14 after transplantation was most severe in 0-24 groups irrespective of the preservation solution. Proteinuria progressed in all groups over the course of the experiment.

Based on compositional differences some specific advantages of IGL-1 over UW-CSS could be expected. The extracellular composition of IGL-1, with a low potassium content, is believed to favour wash-out efficacy resulting in better tissue penetration of the preservation solution. In this study we choose to pre-flush the donor kidney with 0.9% NaCl before flushing it with either IGL-1 or UW-CSS to study preservation capacity without a possible blunting effect of differences in wash-out. We could not demonstate any advantage of the extracellular IGL-1 over the intracellular UW-CSS.

Furthermore, IGL-1 differs from UW-CSS with regards to the used colloid. The feasibility of HES as a colloid in UW-CSS has been extensively debated. HES prevents interstitial edema but also increases viscosity. Analyzing the effect of HES on red blood cells (RBCs), several authors have shown an increased RBC aggregability in both human and rat whole blood when large molecular sized HES is present (9;11). PEG in IGL-1 does not have aggregating effects on RBCs and has immune-modulating properties. In contrast to Hauet and colleagues, who found a marked reduction of inflammatory injury when using a PEG based solution compared to UW-CSS we could not detect any differences in macrophage infiltration (6). There are three possible explanations for the different results. First, we have used an isogenic rat transplant model versus the pig autotransplantation model of Hauet and colleagues. Second, our data on infiltration in the present study was obtained 14 days after transplantation, whereas Hauet and colleagues used biopsies taken after seven days. Therefore, it is possible that we have missed a transient macrophage infiltration. At an earlier timepoint, 4 days post-transplant, we did measure urinary TBARS levels as indicator for increased lipid peroxidation by ROS. Apart from ischemia/reperfusion injury, leukocyte infiltration is an important generator of ROS production (33). We could, however, not detect any differences between IGL-1 and UW-CSS when studying downstream post-infiltration effects such as urinary TBARS levels at day 4. Furthermore, focal glomerular sclerosis and interstitial fibrosis at day 14 post-transplant did not differ between the two solutions. Third and probably most important, the

molecular weight of PEG in IGL-1 is 35 kDa whereas Hauet and colleagues used a 20 kDa PEG in their preservation solution. Therefore, the size of high-molecular weight PEG could be critically important to sterically avoid T-cell activation via immunological synapses (34). We speculate that PEG 35 kDa is too large to interfere in these synapses and limit T-cell activation. When comparing PEG 35 kDa to PEG 20 kDa in a porcine proximal tubular epithelial cell preservation experiment PEG 35 kDa was more potent than PEG 20 kDa in preserving ATP content, reducing LDH release and limiting oxidative stress (34). To have the best of both worlds, the combination of PEG 20 kDa and PEG 35 kDa should be examined in future preservation solutions.

This study has some limitations. We could not study the combination of 15 min of WIT and 24 h CIT in more detail because all animals died of renal failure shortly after transplantation. Therefore we are developing a rat kidney transplantation model with isolated cannulation of the ureter. This will allow selective functional assessment of the transplanted kidney, while a native kidney can remain in situ to sustain adequate dialysis. Since, in our model, serum creatinine levels revealed that adequate kidney function restored within one week after transplantation, future studies need evaluation at earlier timepoints. After two weeks most of the ischemia/reperfusion injury has already been repaired, making it difficult to pinpoint differences between groups.

Overall, there was no significant difference between the used solutions in terms of kidney damage or graft function. Although IGL-1 was expected to have some advantages over UW-CSS based on compositional differences, we were not able to demonstrate any differences between IGL-1 and UW-CSS in this rat transplantation model.

Acknowledgments

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Chapter V

The Groningen Machine Perfusion system: Functional evaluation of a new machine perfusion device

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Abstract

Background: To improve preservation of donor livers, a portable hypothermic machine perfusion (HMP) system was developed as an alternative for static cold storage. A prototype of the system was built and evaluated on functionality. Evaluation criteria included 24 h of adequate pressure controlled perfusion, sufficient oxygenation, a maintained 0-4°C temperature and sterile conditions.

Methods: Porcine livers were perfused with pump pressures that were set at 4 mmHg (continuous flow, portal vein) and 30/20 mmHg at 60 BPM (pulsatile flow, hepatic artery). Control livers were preserved using the clinical gold standard: static cold storage. In the HMP group, pressure, flow and temperature were continuously monitored for 24 h. At time-points 0, 2, 4, 8, 12 and 24 h samples of UW-MPS were taken for measurement of partial oxygen pressure (pO_2) and lactate-dehydrogenase (LDH). Biopsies in every lobe were taken for histology and electron microscopy; samples of ice, preservation solution, liver surface and bile were taken and cultured to determine sterility.

Results: Temperature was maintained at 0-4°C; perfusion pressure was maintained at 4 mmHg and 30/20 mmHg for portal vein and hepatic artery, respectively. Flow was approximately 350 ml/min and 80 ml/min, respectively, but decreased in the portal vein, probably due to edema formation. Arterial pO_2 was kept at 100 kPa. Histology showed complete perfusion of the liver with no major damage to hepatocytes, bile ducts and non-parenchymal cells compared to control livers.

Conclusion: The machine perfusion system complied with our design criteria and will have to demonstrate the superiority of machine perfusion over cold storage in transplant experiments.

Introduction

Liver transplantation is nowadays an effective therapy for patients with end-stage liver disease. Preservation of the liver is routinely realised by static cold storage (CS), allowing transport of the donor liver to the receiving patient. However, in the pioneering years of organ preservation hypothermic (0-4°C) machine perfusion (HMP) was used instead of CS. With the first generation perfusion machines it became possible to preserve kidneys for more than 72 h. Soon after this pioneering work in kidney preservation, liver machine preservation became feasible as well (1;2). Following the discovery of Collins solution, especially the development of the University of Wisconsin preservation solution (UW-CSS) made it possible to better preserve organs statically without machine perfusion and maintain organ viability for transplantation (3;4). The CS preservation technique made organ preservation and transplantation a standard clinical procedure due to its simplicity and low cost. UW-CSS allows CS preservation of donor livers for 12 to 18 h in the clinical setting and even storage beyond 48 h in laboratory experiments (5-7). The success of the UW-concept and the introduction of a number of modifications has significantly contributed to a better understanding of ischemia/reperfusion injury. Nevertheless, the limits of static CS preservation in organ transplantation appear to be reached.

Currently, the majority of donor livers used for transplantation originate from brain-dead donors only. Livers from marginal or donation after cardiac death (DCD) donors are only occasionally used for transplantation due to expected decreased organ viability after transplantation. In kidney preservation, hypothermic machine perfusion is now used in a number of centers for preservation of DCD and so-called marginal donor kidneys (8-11). In the experimental setting, this technique allowed up to 5-7 days successful preservation and transplantation of canine kidneys (12;13). Due to these successes and the potential to increase the donor pool and prolong storage times, continuous preservation of the liver has gained renewed interest. Continuous machine perfusion of the liver could contribute to better preservation of brain dead donor livers, facilitate the use of marginal donor livers and might result in the use of more DCD donors as well (14;15).

Furthermore, HMP could allow the use of 'on-line' viability markers by analysis of pressure and flow characteristics and perfusate. HMP could, thus, allow a refined clinical decision to include or discard marginal and non-heart-beating donor organs in the transplantation procedure.

In the late 1960's Belzer, Slapak and Brettschneider experimented with continuous hypothermic machine perfusion of the liver in an experimental setting with results comparable or even better than livers preserved with static CS (2;16;17). In 1986 D'Alessandro and later Pienaar and colleagues managed to transplant good quality canine livers after 72 h preservation in a HMP dog model (18).

However, despite these successes, until now a clear definition and validation of optimal perfusion settings have not become available. Previously, our group reviewed the HMP literature for liver, and defined three determining factors for effective HMP: the type of preservation solution, the characteristics of perfusion dynamics, and the

necessity for oxygenation (19). It was concluded that, to date, Belzer's University of Wisconsin machine preservation solution (UW-MPS) is the most suitable perfusate. Perfusion through the portal vein alone has been shown to result in good short term liver viability upon transplantation (20-23).

Nevertheless, to improve single blood vessel perfusion, simultaneous perfusion of portal vein and hepatic artery, might prevent ischemic-type biliary lessions leading to biliary strictures and retransplantation (24). Data about which pressure and flow should be used differ between authors, but there is agreement about the use of a lower than physiological perfusion pressure and flow during HMP to limit any shear stress induced endothelial cell injury. Based on an educated guess and proved by our own experiments comparing different perfusion pressures using fluorescence microscopy, it was found that a portal venous perfusion pressure of 4 mmHg and a pulsatile hepatic arterial pressure of 30/20 mmHg (systolic/diastolic) at 60 BPM resulted in a complete and uniform perfusion of the liver with minimal endothelial and/or hepatocyte damage (25). Previously, many authors have pointed at the potential importance of additional oxygenation during liver HMP (24;26-30). Based on the data of Fuija and colleagues as well as our experiments the required partial oxygen pressure was determined at 55 kPa to comply with total liver oxygen demand during HMP (28;30). In addition to an adequate perfusion, it was concluded that the hypothermic liver uses oxygen and thus oxygenation of the perfusate, even at low temperatures, remains necessary. Physiological oxygen distribution in the liver is 65% through the hepatic artery and 35% through the portal vein. Mimicking this situation in our HMP system, a pO_2 of 35.8 kPa and 19.2 kPa is needed in the hepatic arterial and portal venous line, respectively.

So far, there is no clinical liver perfusion machine commercially available to improve organ viability and seriously challenge the limits of liver preservation by optimizing perfusion and transportation during cold storage, comparable to machine preservation of kidneys in dedicated transplant centers.

Therefore, a hypothermic liver perfusion machine using oxygenated UW-MPS and dual vascular perfusion technique that is able to perfuse the liver for 24 h and attempts to maintain the quality of donor livers was developed. The ultimate clinical goal with this machine is to obtain an increase of the donor pool, maintain viability and prolong storage times. The aim of the study presented here was to test the technical performance of a prototype of the Groningen Machine Perfusion (GMP) system. This includes adequate perfusion of the liver (i.e. uniform perfusion with no or minor cellular injury), maintenance of hypothermia, pressure and sterility, and supply of the liver tissue with sufficient oxygenation during 24 h of continuous preservation. Morphological changes after preservation were compared to histology after preservation using the clinical gold standard: static cold storage.

Animals, materials and methods

Animals

Eight female pigs weighing 40-50 kg were used. A porcine model was chosen since pigs are phylogenetically more similar to humans than rodents or other domestics. All experimental procedures were approved by the Animal Experiments Committee of the University of Groningen. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed.

Experimental design

The following groups were studied:

- Group 1: 24 h HMP using the GMP system (Fig. 1, n=6);
- Group 2: 24 h CS using UW-CSS (n=2).

The experiment (Fig. 2) started with the procurement and blood wash-out of the donor liver. After procurement, the liver was submerged in the reservoir, containing UW-MPS and the portal vein, celiac trunc and common bile duct were cannulated. The sterilized disposable unit, including the reservoir, pump heads, oxygenator and tubing was subsequently attached to the HMP system. The perfusion pressure was set at 4 mmHg and 30/20 mmHg for portal vein and hepatic artery, respectively. After 24 h perfusion was stopped and the liver was taken out of the reservoir. Just prior to and after preservation, biopsies of the liver were taken for histology. During preservation, at time points 0, 2, 4, 8, 12 and 24 h, samples of the perfusate were taken to assess the partial oxygen pressure and lactate-dehydrogenase (LDH) levels.

In contrast to the experimental group, livers in the control group were submerged in University of Wisconsin cold storage solution (UW-CSS) and subsequently stored on ice. Biopsies were taken before and after preservation.

The performance of the GMP system was assessed by judging:

1. perfusion dynamics, with a controlled constant pressure and resulting flow, maintained temperature at 0-4°C, and uniform perfusion without loss of tissue integrity as compared to controls.
2. oxygenation, showing pO_2 > 35.8 kPa and 19.2 kPa for hepatic artery and portal vein, respectively.
3. microbiology, to demonstrate sterility inside the disposable unit throughout the entire preservation period.

Groningen Machine Perfusion (GMP) system

The GMP system consists of a reservoir in which the liver is placed, two miniature centrifugal pumps (Deltastream DPII, MEDOS Medizintechnik AG, Stolberg, Germany) delivering continuous and pulsatile flow, respectively, a miniature hollow fibre membrane oxygenator (HILITE 800LT, MEDOS Medizintechnik AG, Stolberg, Germany), an oxygen cylinder, a battery pack and a measurement and control unit which is connected to a handheld computer interface (Fig. 1). The pulsatile pump

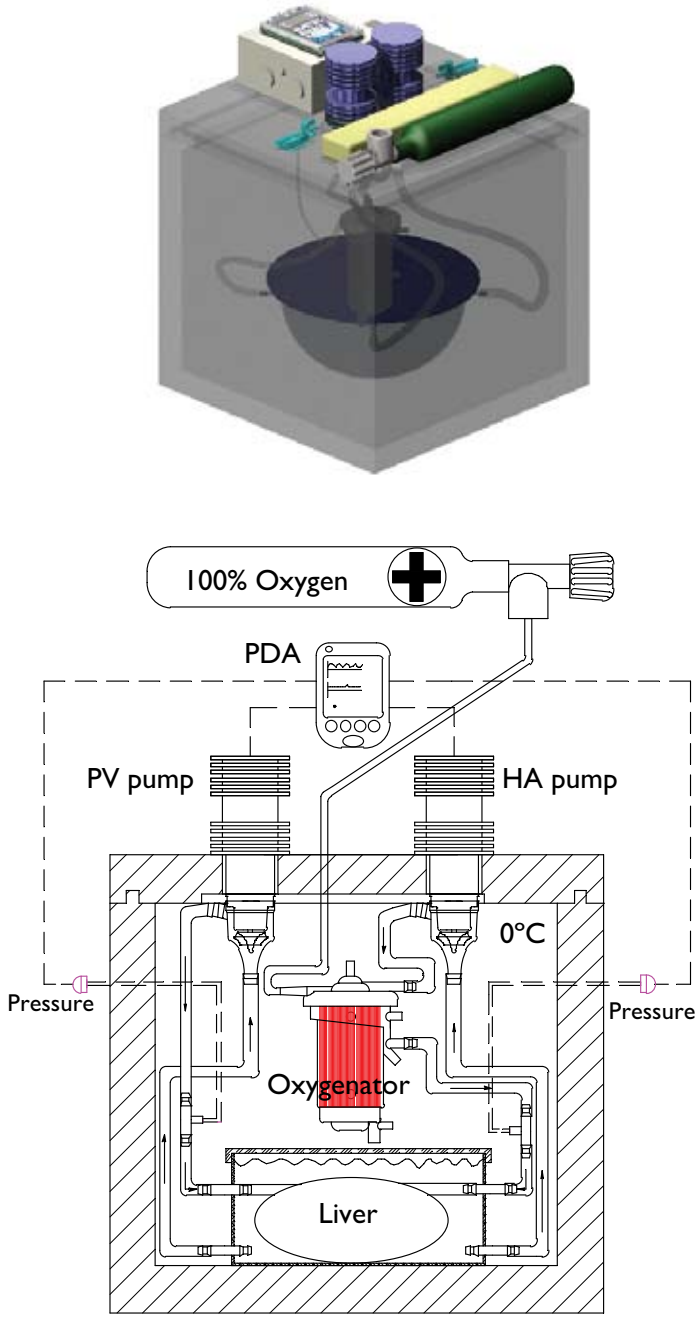

Figure 1. Prototype of the Groningen Machine Perfusion (GMP) system for liver preservation.

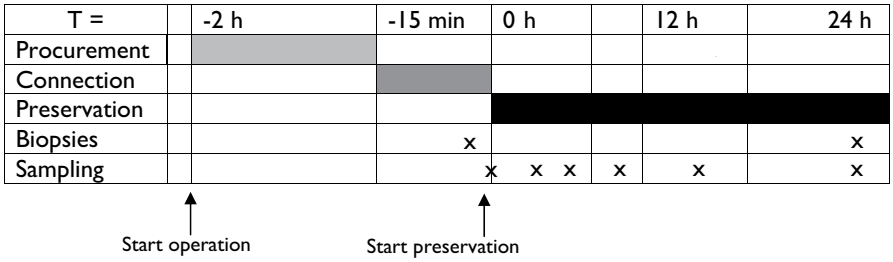

Figure 2. Experimental design.

directs the preservation solution (UW-MPS) from the reservoir through the oxygenator (O_2-flow 300 ml/min) the hepatic artery; the continuous pump perfuses the portal vein without oxygenation. Both pumps are pressure-controlled, and recirculate the preservation solution with a constant perfusion pressure. The sterile disposable unit of the GMP system, including the reservoir, pump heads, oxygenator, cannulas and tubing, is situated inside a polystyrene cooling box (Wolters kunststoffen, Enter, The Netherlands) which is filled with melting ice to secure a hypothermic temperature of 0-4°C for more than 24 h.

Operative procedure

After anaesthesia using 10 mg/kg ketamine i.m., the animal was intubated and mechanically ventilated with a mixture of oxygen and isoflurane. The procurement of the liver followed the standard techniques for human multiorgan retrieval (31). The same donor procedure was applied for both experimental and control livers. Briefly, the donor operation was performed through a midline laparotomy. The abdominal aorta was isolated and encircled with a ligature distally of the renal artery for insertion of an infusion cannula (18 Fr, Tyco Healthcare, Zaltbommel, The Netherlands). The aorta was dissected from the surrounding tissue cranially of the celiac trunc to allow crossclamping during infusion of lactated-Ringer's solution. The supra- and infrahepatic vena cava were dissected and prepared for ligation. The splenic and left gastric arteries were dissected and ligated. The celiac trunc was dissected free from its surrounding tissue towards the aorta. The common bile duct was mobilized and transected. The portal vein was then freed and prepared for cannulation. At this point the pig was fully heparinized (20.000 units), the aorta was crossclamped cranially of the celiac trunc and was subsequently cannulated caudally of the renal artery followed by infusion of lactated-Ringer's solution. After three liters of aortic infusion a cannula (18 Fr, Tyco Healthcare, Zaltbommel, The Netherlands) was placed into the portal vein and infused with one liter of lactated-Ringer's solution. The intrapericardial inferior vena cava was transected (31;32). The celiac trunc was dissected in continuity with a circumferential piece of aorta.

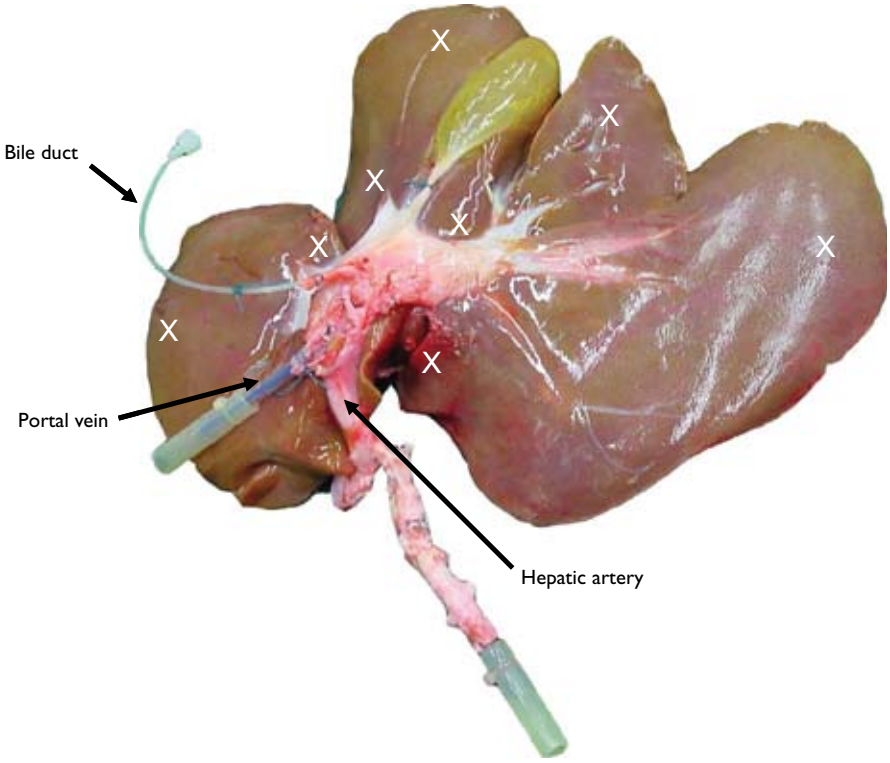

Figure 3. Porcine liver indicating cannula positions and biopsy locations (X).

Back table procedure

Cannulation with an 18 Fr cannula of both the portal vein and aortic stump was performed with the liver submerged in 4 liters 0-4°C UW-MPS. The cystic duct was ligated and a catheter (6 Fr) was placed in the common bile duct for decompression and collection of bile formed during preservation (Fig. 3). At this point samples and biopsies were taken for microbiological examination, histology, electron microscopy (EM) and fluorescence microscopy (FM). Biopsies were taken in the liver hilus and periphery in four liver lobes, i.e. right medial lobe, right lateral lobe, left medial lobe, left lateral lobe (Fig. 3). A peripheral biopsy in the left lateral lobe was used for EM. Acridine Orange (AO: 5 mg/l) and Propidium Iodide (PI: 10 mg/l) were subsequently added to the UW-MPS.

In control livers biopsies were taken at the same locations described above. As in the experimental group AO and PI were added to the UW-CSS solution.

Data collection

In the experimental group perfusion pressure (Truwave, Edwards Lifesciences, Irvine, USA), flow rates (H7C, Transonic Systems, Ithaca, USA) and temperature (10k3D(X),

BetaTherm, Galway, Ireland) were continuously recorded for 24 h. Samples of the perfusion solution were taken at 0, 2, 4, 8, 12 and 24 h of machine perfusion. LDH as a marker for liver damage, and partial oxygen pressure (pO_2) were measured in these samples.

In both groups liver biopsies (Fig. 3) were taken after 24 h of preservation close to the previous location to determine tissue integrity using light and fluorescence microscopy. For the experimental group biopsies for electron microscopy were additionally taken.

Microscopic techniques

Cryosections (4 µm) were examined to identify the location and amount of dead cells, stained with the exclusion-dye PI and to disclose minute staining with AO. A fluorescence microscope (Leica DC300F, Rijswijk, The Netherlands) with 495/519 nm (FITC) and 547/572 nm (TRITC) filter was used, with a magnification of 20 times. PI-positive cells were computer-counted in a blinded fashion using 4 microscopic fields at a magnification of 10 times. Light microscopy (magnification 20 times) of haematoxylin and eosin stained sections was used to demonstrate changes in morphology. Tissue was collected, fixated in 4% formalin, subsequently paraffin embedded and cut into 4 µm thick sections.

Microbiology

Samples of perfusion solution, liver, bile and ice, taken before and after 24 h machine preservation, were smeared on blood agar plates and cultured at 37°C in aerobic and anaerobic environments for seven days. The agar plates were subsequently macroscopically examined for colony forming bacteria and hence bacterial contamination.

Statistics

Statistical analyses were performed using Repeated Measures ANOVA for data measured on the same liver at different time steps and Wilcoxon signed ranks tests for histological evaluation. For all analyses, a p-value ≤ 0.05 was considered to indicate statistical significance. Results are expressed as the mean ± the standard error of the mean (SEM).

Results

Perfusion dynamics

Temperature measurements showed a constant temperature inside the cooling box of 0°C at time points 0, 2, 4, 8, 12 and 24 h for all experiments.

Mean hepatic arterial perfusion pressure was constant (25 mmHg) (Fig. 4) at these time points. Portal venous perfusion pressure increased gradually over time starting after 12 h. This increase was, however, not significant.

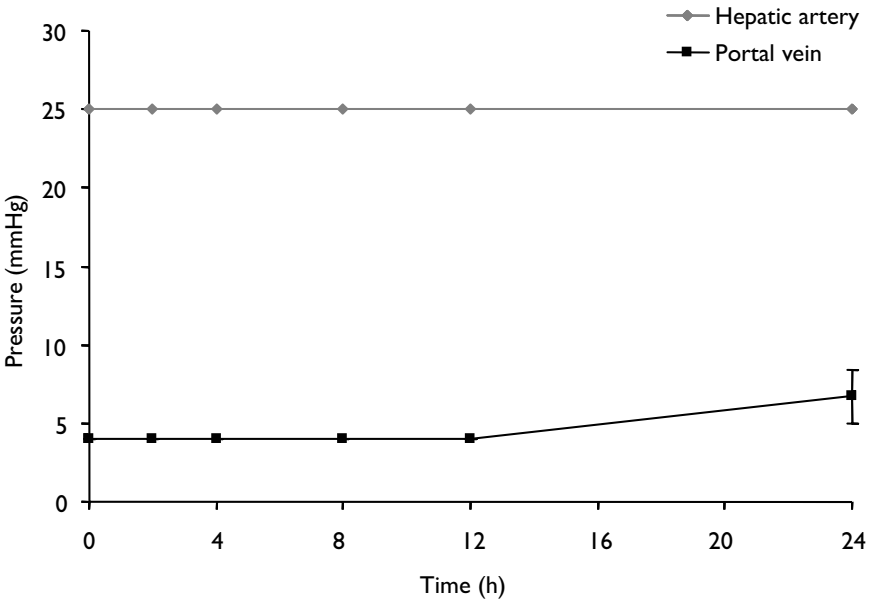

Figure 4. Mean perfusion pressure during 24 h HMP (mean ± SEM). No significant increase of pressure was observed.

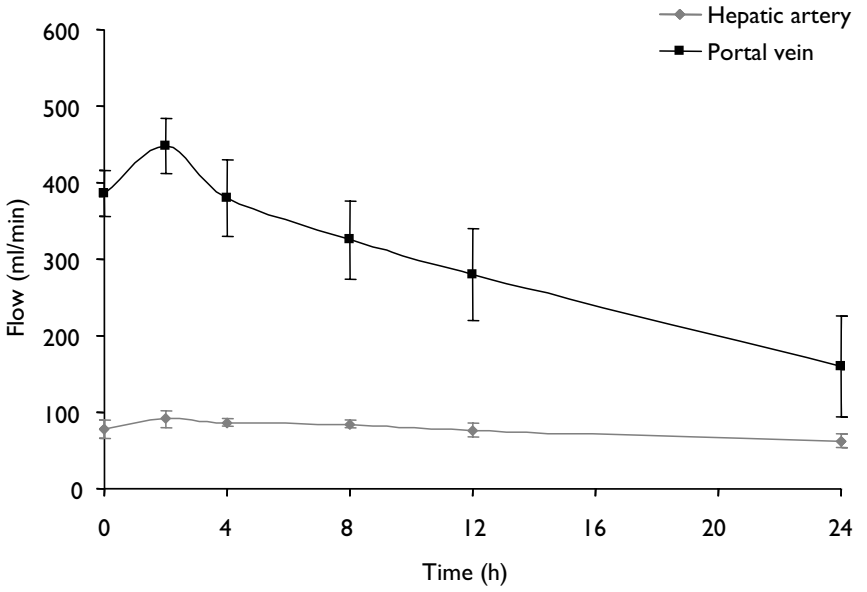

Figure 5. Flow during 24 h HMP (mean ± SEM). A significant decrease in both portal venous and hepatic artery flow was seen over time.

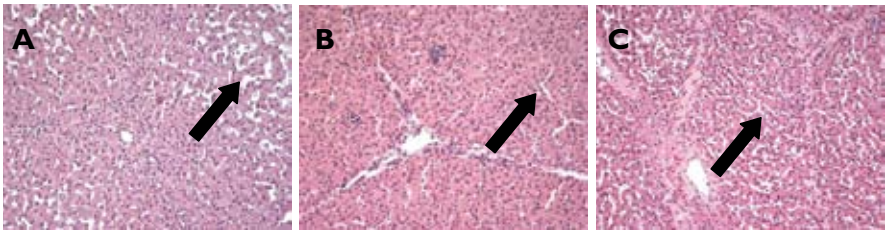

Figure 6. Widened sinusoids (black arrows) before **(A)** and after 24 h of HMP **(B)** or CS **(C)** preservation shown with light microscopy using hematoxyline-eosine staining. Compared to CS (right image) no extra morphologic damage could be demonstrated in the HMP group. (Magnification 20x).

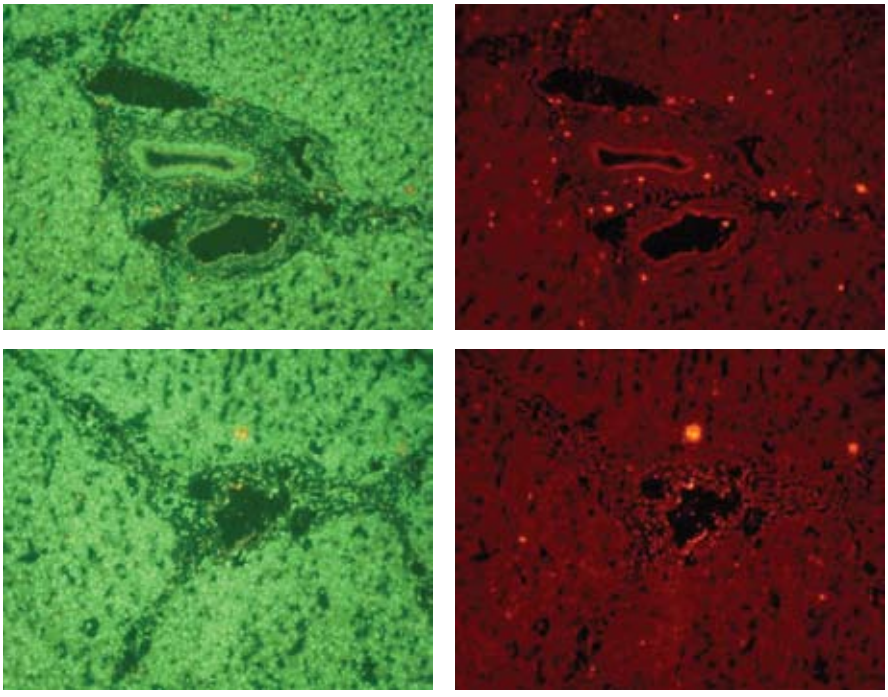

Figure 7. Rate of perfusion (left) and number of dead cells (right) after 24 h HMP, stained with acridine orange (AO, left) and propidium iodide (PI, right), respectively, for the liver hilus (top) and periphery (bottom). In control CS grafts no staining was detectable (data not shown). (Magnification 20x, Fluorescence microscopy 455/519 nm (FITC) and 547/592 nm (TRITC) filter).

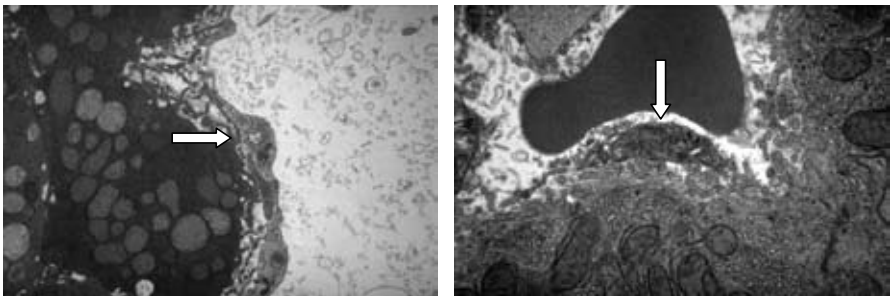

Figure 8. Sinusoidal endothelial cells (white arrows) before (left) and after (right) 24 hours of HMP. (Transmission electron microscopy, magnification 25000x).

Hepatic arterial flow measurements showed an initial increase in the first two hours of preservation, from 78 ± 12 ml/min to 91 ±11 ml/min (Fig. 5). After two hours, the mean hepatic arterial flow decreased gradually from a maximum of 91 ± 11 ml/min to a minimum of 63 ± 8 ml/min after 24 h of machine perfusion ($p<0.05$). A similar trend was visible in the portal venous flow, with a small rise between 0 and 2 h (from 386 ± 30 ml/min to 448 ± 35 ml/min, respectively) and a subsequent gradual decrease to 160 ± 65 ml/min after 24 h ($p<0.05$).

Histological evaluation of biopsies taken from hilus and periphery revealed expanded sinusoidal spaces after 24 h of machine preservation (Fig. 6). At the start of preservation, widened sinusoids, indicating congestion, were already visible. No differences could be observed in comparison to the control group.

In the experimental group, fluorescence microscopy resulted in a uniform staining pattern in both hilus and periphery (Fig. 7). Staining with propidium iodide (PI), showing the number of dead cells, revealed significantly more PI-positive cells in the liver hilus compared to the periphery. Hilar counts of PI-positive cells showed 66 ± 14 PI-positive cells per microscopic field versus 24 ± 5 PI-positive cells in the periphery ($p<0.05$). The dead cells found were determined to be non-parenchymal cells, i.e. remaining blood cells, Kupffer cells and endothelial cells. In the control group no staining with AO or PI could be detected.

Finally, electron microscopy was conducted to evaluate the condition of the sinusoidal endothelial cells (Fig. 8) after HMP. In control biopsies, the endothelial cells showed their characteristic orientation, with stretched cytoplasm along the sinusoids. After 24 h of HMP, endothelial cytoplasm retracted, creating fenestrations between the endothelial cells. The cellular nuclei were still intact.

LDH released in the perfusate is a marker for cellular injury and showed a significant increase from 102 ± 12 U/l to 148 ± 20 U/l over the total preservation period in the HMP grouop (Fig. 9). Due to its static character, no LDH data was available for the CS group.

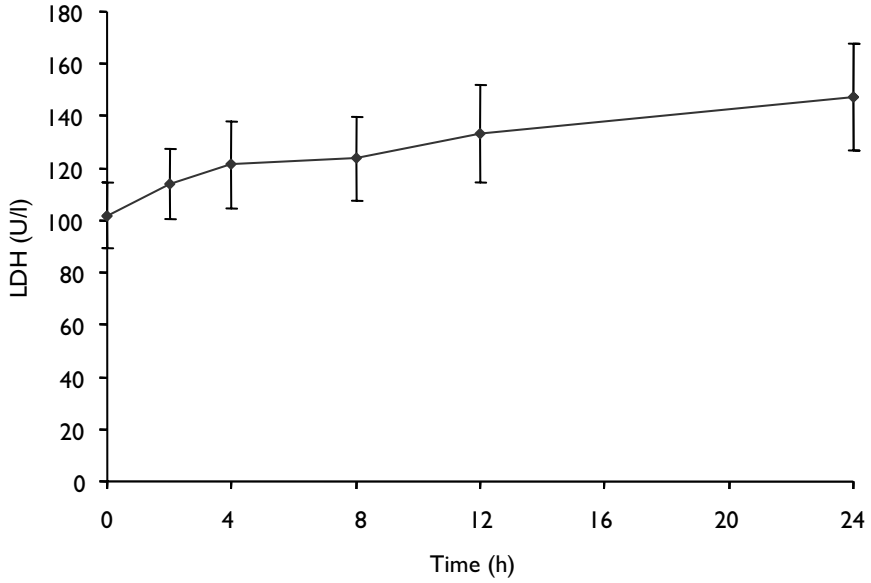

Figure 9. Significant increase of lactate-dehydrogenase (LDH) levels during 24 h HMP (mean ± SEM).

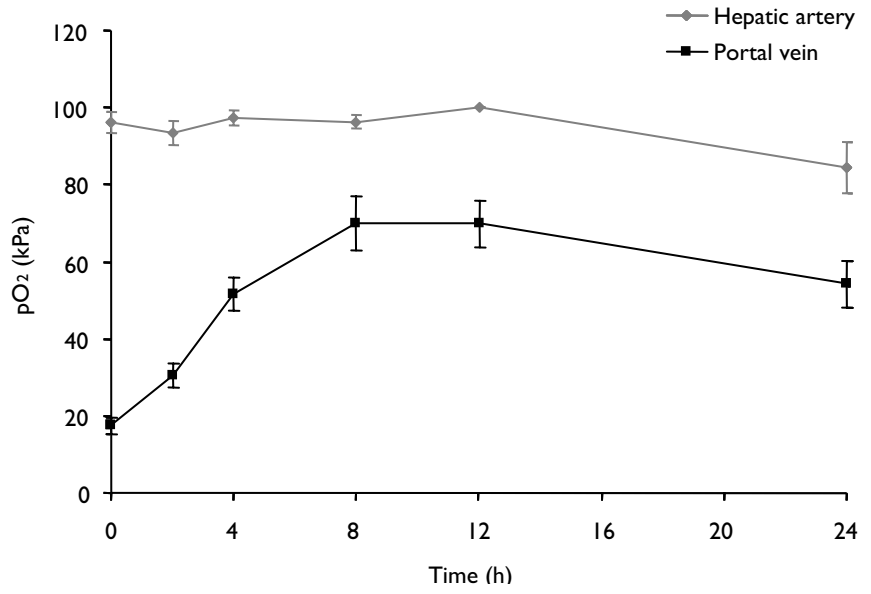

Figure 10. Partial oxygen pressure in the hepatic arterial line and the portal venous line (mean ± SEM). While partial oxygen pressure remained constant in the hepatic artery line, a significant increase was seen in the portal vein line during the first 8 h of HMP.

Oxygenation

The pO_2 was constant (95 ± 3 kPa), in the arterial line, in which the oxygenator is situated (Fig. 10). The partial oxygen pressure in the portal venous line, which is not directly oxygenated, was initially low (17 ± 2 kPa), but increased significantly to a mean pO_2 of 65 ± 6 kPa after 8 h and remained constant until 24 h.

Microbiology

Samples obtained at 0 h and prior to preservation from liver, bile and perfusion solution showed no bacterial colonies after seven days of culturing. In comparison, as expected, non-sterile ice samples, however, were found to be largely contaminated with bacteria. However, after 24 h of preservation, both the perfusion solution and the liver remained sterile.

Discussion

Hypothermic machine perfusion preservation of kidneys is believed to improve preservation quality and result in longer preservation times and a reduced incidence of delayed graft function (18;33;34). As a consequence, HMP allows inclusion of DCD donors in the kidney donor pool. To extend these advantages to the liver donor pool, many authors have been studying HMP of the liver as well (14;15;17;18;21;22). 72 h successful preservation of good quality dog livers has been reported, and the first promising experiences with HMP of DCD donor livers in pigs and rats have been documented (14;15;35). However, despite these successes, liver HMP has not become the clinical standard preservation procedure yet. One of the reasons for this is that current machines are non-transportable and/or not easy to use without a stand-alone possibility requiring continuous supervision.

The GMP system constitutes an attempt towards introduction of machine preservation for the liver into clinical practice. New developments and techniques allow expansion of the conventional static cold storage procedure to a portable continuous perfusion system using miniaturized blood pumps, small oxygenators and micro-electronics. Design criteria for the pump system included pressure-controlled oxygenated perfusion with arterial and portal venous pressures that are high enough to enable a uniform perfusion throughout the liver without compromising endothelial cell integrity. Additional design criteria included a continuous cold temperature and sterility for the duration of 24 h.

The prototype of the GMP system is able to maintain a temperature of 0-4°C. The cooling box in which the reservoir, oxygenator and pump heads are located, was filled with melting non-sterile ice and proved to be able to maintain low temperatures without refilling. The disposable unit remains sterile since neither aerobic nor anaerobic bacterial colonies were found in the perfusate after 24 h preservation. The control

unit manages to maintain hepatic arterial pressure at 30/20 mmHg for 24 h, while portal venous pressure will increase to a certain extend after 12 h of perfusion. During the total preservation period portal venous flow varied considerably, which was a result of altered resistances in the liver. At 24 h, portal venous resistance was at such a level that the controller could not maintain a perfusion pressure of 4 mmHg, since the minimal rotational speed of the centrifugal pump was reached.

During the first 12 h of the preservation period, the controller maintained a perfusion pressure of 4 mmHg with an initial flow of 448 ml/min to a final flow of 160 ml/min at 24 h. Since perfusion pressure is maintained, flow variation resembled vascular resistance, defined as perfusion pressure divided by resulting flow. This variation of mainly portal venous flow was unexpected and, according to our knowledge, it has never been reported in literature before. Yamamoto continuously perfused porcine livers through the portal vein, but found no increase in resistance during 72 h of machine preservation (23). The increase in resistance could have been caused by interstitial edema formation. However, light microscopy did not reveal major edema formation in the microvascular system of the liver. Some spacing of the sinusoids was seen, both in the HMP and CS livers. This phenomenon has been described before in hypothermic preservation for both cold storage and machine perfusion (33;36). In this first try-out experimental series edema formation in the connective tissue of the extrahepatic portal vasculature was found. The extent of perivascular edema could explain the increased flow resistance, and the subsequent decrease in flow. This finding might be the result of a still too high perfusion pressure.

Using static cold storage, pig livers have been preserved for 6-8 h and transplanted with good survival rates, while 50-80% of the animals die when transplanted after 10-12 h preservation (37). With 24 h of HMP, the maximum viable preservation time of the pig liver could have been reached, reflected by the increased resistances. Whether the cause of the decreasing flow during our 24 h HMP is related to a high perfusion pressure or to other species-dependent effects remains to be investigated. The consequences for liver viability are unknown, since no further major damage to the liver was seen. Future experiments in a large animal liver transplantation model will, therefore, be performed.

HMP as such could induce shear stress to the endothelial cells which are already more sensitive at low temperature and thus prone to injury (38). Microscopic observation and computer counting of PI-positive cells showed significantly more damaged cells in the hilum of the liver compared to the periphery, indicating that the perfusion pressure was chosen too high. PI-positive cells were found to be of non-parenchymal origin and located near the portal triad of the liver lobules. Comparison to CS livers could not be made due to lack of AO and PI staining during CS. Probably, the relative short period of intravascular flush does not allow adequate staining with either AO or PI. The extent of damage to the endothelial cells in the HMP group was studied in more detail using electron microscopy. The endothelial cell structure before HMP and after initial flushing was normal with a cytoplasm stretched along the sinusoidal wall and aligned

to hepatocytes. After 24 h of HMP, the nuclei of the endothelial cells were still intact, but the long-stretched cytoplasm was now retracted. This result has been confirmed by other authors who also measured decreased function of endothelial cells after HMP (33;36). Shear stress-induced endothelial cell injury could be prevented by lowering the perfusion pressure. However, staining with acridine orange indicated that the perfusion pressure was well chosen to obtain a complete and uniform perfusion of the liver. Lowering the perfusion pressure could result in a lower incidence of endothelial damage but in an incomplete perfused liver.

Another important design criterion for the GMP system was to obtain sufficient oxygenation. The hollow fibre membrane oxygenator placed in the hepatic arterial line managed to saturate the perfusate immediately after starting HMP. The pO_2 in the portal venous line, without oxygenator, increased slowly from room-air saturation to 65 kPa after 8 h and remained then constant during preservation. The oxygen pressure complied to the design criterion for oxygenation, for both lines. The difference between arterial and portal venous partial oxygen pressures was the amount of oxygen consumed by the liver itself, compensating for the saturation time of the total reservoir. After 8 h reservoir saturation was reached and oxygen uptake by the liver remained constant but still considerable (35 kPa). This finding is in accordance with other authors, who showed an improved liver viability after oxygenated vs. non-oxygenated HMP indicating that the liver, even under hypothermic conditions, has a considerable oxygen demand (26-28).

The injury marker LDH increased during the preservation period. Considering the cumulative measurement of LDH, this increase was expected. Combining this finding with the low amount of damaged hepatocytes, indicated with fluorescent PI staining and electron microscopy, it is concluded that the perfusion regime of the GMP system did not induce any major injury to the pig liver. However, liver viability can only be judged in a reperfusion model, i.e. either an in vitro isolated reperfused liver model or ultimately a transplantation model.

In summary, the GMP system prototype was able to dually perfuse the liver for 24 h in a pulsatile (HA) and continuous (PV) manner. The system maintained adequate flow at a controlled pressure. Although an increasing resistance of the liver was observed, no major endothelial cell or hepatocyte damage was induced compared to CS. Some perivascular extrahepatic edema was, however, found. This phenomenon could either be pressure-induced or species-specific, which will be investigated in another large animal model. The hollow fibre oxygenator oxygenated the arterial line satisfactory, and even supplied the portal venous line with a sufficient amount of oxygen. Melting non-sterile ice kept the temperature of the disposable section, including reservoir, oxygenator, pump heads and liver, below 4°C during 24 h, and did not affect the sterility inside the disposable section.

The GMP system has proven to comply with the design criteria and requirements that were formulated to optimally preserve a donor liver. Following these functionality

tests, the system now must demonstrate the advantages of hypothermic dual perfusion of the liver over cold storage in a transplant model.

If correct, the system allows extended preservation times despite lower quality of donors. This would make liver transplantation a semi-emergency procedure. Preservation outcome would then be improved in such a way that the incidence of initial poor function and primary non-function could decrease and in addition, even DCD donor livers ought to be included in the donor pool on a more regular basis.

Acknowledgments
We would like to acknowledge the entire technician team of the Experimental Animal Facility.

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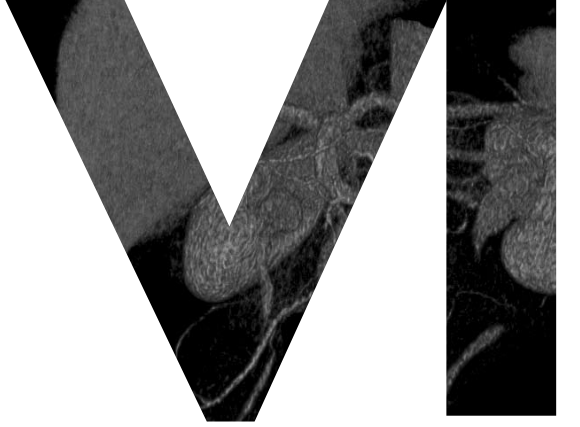

Chapter VI

Improved kidney graft function after preservation using a novel hypothermic device: the Groningen Machine Perfusion system

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Abstract

Background: The increasing proportion of marginal donors and donation after cardiac death necessitates better preservation methods to maintain adequate graft viability. Hypothermic machine perfusion (HMP) is a promising alternative to static cold storage (CS). Therefore, we have developed and tested a HMP device which is portable and actively oxygenates the perfusate via an oxygenator. The aim of the present study was to examine the efficacy of the Groningen Machine Perfusion (GMP) system in a kidney transplantation experiment.

Methods: In a porcine autotransplantation model kidneys were retrieved and either cold stored in UW-CSS for 20 h at 4°C or subjected to HMP using UW-MPS at 4°C with two different pressure settings: 30/20 mmHg or 60/40mmHg.

Results: HMP at 30/20 mmHg was found to better preserve the viability of kidneys reflected by improved cortical microcirculation, less damage to the proximal tubule, less damage mediated by reactive oxygen species, less pro-inflammatory cytokine expression and better functional recovery after transplantation. However, high perfusion pressures (60/40 mmHg) resulted in higher expression of von Willebrand factor and monocyte chemotactic peptide-1 in post preservation biopsies and subsequent graft thrombosis in two kidneys.

Conclusion: It is concluded that the GMP system improves kidney graft viability. Perfusion pressures during HMP are, however, critically important for outcome.

Introduction

Maintaining organ viability between donation and transplantation is of critical importance for optimal graft function and survival. To date, static cold storage (CS) is the most widely practiced method in organ preservation. Although simple and effective, this method may no longer be sufficient to prevent further deterioration of the graft-to-be in an era with increasing numbers of older, more marginal and even donation after cardiac death (DCD) donors. An alternative method to maintain donor organ viability is hypothermic machine perfusion (HMP). HMP is, however, not a frequently used clinical preservation method. Based on data from the Organ Procurement and Transplantation Network (OPTN) as of October 2006, only 20% of all kidneys in the United States are preserved using HMP. Although no accurate data are available, the numbers in Europe, by tradition, will be lower compared to the US. In kidney preservation, both animal experiments and historical controlled clinical studies have demonstrated that HMP yields better early graft function compared to static CS (1;2). Most HMP devices in these studies, however, do not actively oxygenate the perfusate which has been demonstrated to be an important additional feature of HMP by a number of groups (3;4). Also, and despite of the fact that HMP was introduced in the late sixties, uncertainty has remained about the optimal pressure settings which vary per series between 30 and 60 mmHg systolic and 6 to 40 mmHg diastolic pressure (5-12). While low pressure settings could underperfuse the kidney, especially the cortex, and lead to a higher delayed graft function, higher arterial pressures have been shown to enhance shear stress and damage the endothelium. To be able to clinically use HMP within an international organ sharing system, as in Europe, and still benefit from its advantages as described by Belzer and others (13;14), a novel HMP device for kidney preservation was developed. This compact, simple portable pulsatile hypothermic perfusion system incorporates advanced miniature technology and actively oxygenates the perfusate. It consists of a disposable unit which fits into the conventional polystyrene box as used in static cold storage preservation.

After completion of the in vitro and liver preservation experiments we have now tested the Groningen Machine Perfusion (GMP) system in a large animal model comparing oxygenated HMP to CS in kidney transplantation. To detect the optimal perfusion pressures we have evaluated both low pressure settings (30/20 mmHg) and high pressure perfusion (60/40 mmHg) at 60 beats per minute (BPM). As an additional focus of this study we have also assessed the extent of reperfusion injury using urinary biomarkers and possible vascular damage during HMP.

Animals, materials and methods

Animals

All experiments were performed in accordance with federal legislation regarding the protection of animals. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed.

Commercially available female German Landrace pigs, weighing 20 to 30 kg, were housed in metabolic cages. They were fed ad libitum on a constant nutrient laboratory grower diet and were allowed free access to tap water. All animals demonstrated normal renal function before the start of the study.

Experimental Design

The model used was a modification of a well-established porcine renal autotransplantation technique, which is a model for heart beating donation with minimal warm ischemia (1;15). Pigs underwent a left nephrectomy, after which this kidney was preserved for a period of 20 h by either static CS or HMP using two different pressure settings. Following preservation, the left kidney was autotransplanted and the right kidney was removed. Three experimental groups were studied:

- Group 1: CS (n=5);
- Group 2: HMP 30/20 mmHg, at 60 BPM (n=5);
- Group 3: HMP 60/40 mmHg, at 60 BPM (n=5).

Anesthetic protocol

The anesthetic protocol was identical for nephrectomy and autotransplant operations. Animals were pre-medicated with ketamine (10 mg/kg), xylazine (2 mg/kg) and atropine (10 µg/kg) intramuscularly. General anesthesia was induced with midazolam (0.3 mg/kg), pancuronium (0.15 mg/kg) and fentanyl (10 µg/kg) intravenously (IV). After endotracheal intubation using a 7 mm endotracheal tube, anesthesia was maintained with isoflurane 1-5% and oxygen administered at 2 to 4 l/min. Animals were monitored intra-operatively by means of pulse oximetry using a tail probe.

Kidney recovery and storage

Surgery started with cannulation of the right internal jugular vein with a polyethylene (PE) catheter for infusion and collection of blood samples. Via a midline approach, a left nephrectomy was performed. The renal artery was cannulated immediately after procurement. Kidneys were flushed at a pressure of 100 cm H_2O (gravity) with 250 ml of University of Wisconsin cold storage solution (UW-CSS) at 4°C in the CS group or UW-machine perfusion solution (UW-MPS) at 4°C in the HMP groups.

Groningen Machine Perfusion System

After flush out HMP grafts were connected to the GMP system (16). This system consists of a disposable reservoir in which the kidney is placed (organ chamber), a miniature magnetic rotary pump (Deltastream DPII, MEDOS Medizintechnik AG, Stolberg, Germany) delivering pulsatile flow, a miniature hollow fibre membrane oxygenator (HILITE 800LT, MEDOS Medizintechnik AG, Stolberg, Germany), an oxygen cylinder, a battery pack and a measurement and control unit which is connected to a palmtop computer (Fig. 1). The pulsatile pump directs the preservation solution (UW-MPS) from the organ chamber through the oxygenator and into the renal artery. Oxygen flow at 100 ml/min resulted in a pO_2 > 100 kPa in the venous effluent. The pump is pressure-controlled, and re-circulates the preservation solution with constant intra-renal

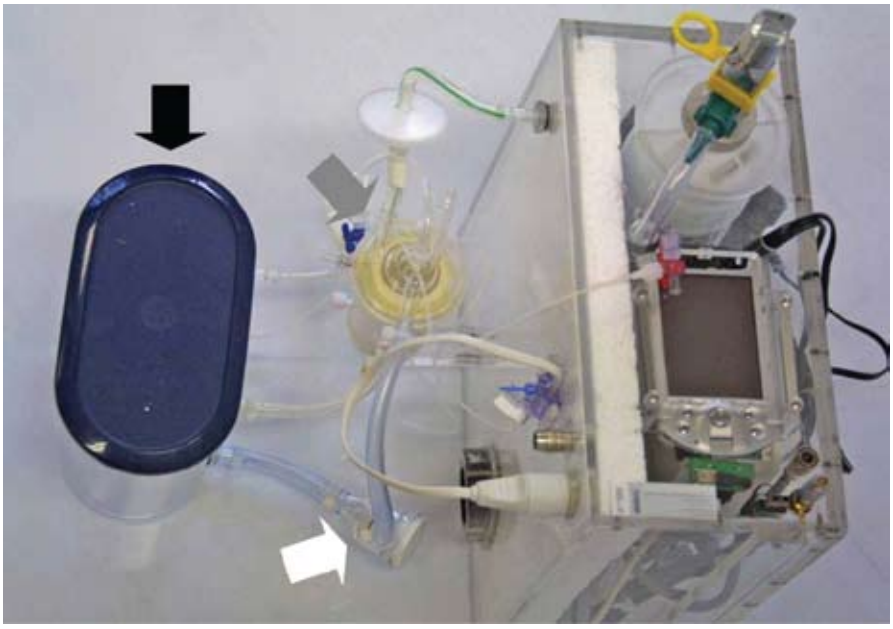

Figure 1. Prototype of the Groningen Machine Perfusion (GMP) system for kidney preservation. On the left side the organ chamber (black arrow), oxygenator (grey arrow) and centrifugal pumphead with click-on system (white arrow). On the right side the oxygen cylinder and Personal Digital Assistant (PDA).

perfusion pressures (30/20 mmHg or 60/40 mmHg) at 60 BPM. Perfusion pressures were corrected for cannula resistance in each experiment. Perfusion flow rates were continuously monitored using clamp-on flow probes (H7C, Transonic Systems, Ithaca, USA). The GMP system is situated inside a standard polystyrene organ box (Wolters kunststoffen, Enter, The Netherlands), filled with melting ice to guarantee hypothermia for more than 24 h (17).

Renal autotransplantation

After induction of general anesthesia the abdomen was re-opened, the right kidney was removed and the graft was transplanted orthotopically. Prior to reperfusion, the graft was flushed with 250 ml of 0.9% NaCl at 4°C to wash out preservation solution. Vascular anastomoses were performed end-to-side (left renal vein – vena cava) and end-to-end (left renal artery – right renal artery), respectively, using 6-0 polypropylene running sutures. The ureter was cannulated using polyethylene tubing which was subsequently tunnelled through the abdominal wall, allowing continuous visual inspection of urine production. Reperfusion was established by release of the venous and arterial clamps, followed by infusion of 100 ml glucose 50% to induce osmotic diuresis. 10 min after reperfusion, renal tissue perfusion was assessed non-invasively as mean erythrocyte flux, determined by Laser Doppler flowmetry (blood

flow monitor DRT4, Moor Instruments, Axminster, England) as described in detail previously (4;18). To account for temporal variations in blood flow, we calculated the mean flux value over 10s of recording, and in order to eliminate the influence of regional heterogeneity, measurements were performed at three distinct locations on the renal surface. All flux measurements were expressed as percent variation from the baseline value obtained from the non-ischemic native kidney in each individual animal.

Postoperative care

Free access to tap water was allowed immediately and supplemented with 2 litres of 0.9% NaCl solution IV during the first 24 h. Standard food was offered on the first postoperative day. Anti-thrombosis therapy was provided by 1 gram acetylsalicylic acid on a daily basis. Antibiotic treatment consisted of perioperative and subsequent administration of 500 mg ampicillin IV on a daily basis. Venous blood samples were taken from the jugular catheter for measurement of renal function in terms of serum creatinine and electrolytes. The jugular catheter was flushed with heparin (3000 IU) after each sample. Daily urine output was measured and urine samples were taken. Animals showing any signs of distress or lethargy because of uremia were sacrificed by lethal injection of T61 (Intervet, Munich, Germany) IV. Surviving animals were sacrificed by injection of T61 on postoperative day 7, after removal of the transplanted graft under general anesthesia.

Tissue preparation and biochemical analysis

Kidney biopsies

Cortical renal biopsies were taken at different stages: before explantation (control), after preservation prior to transplantation and 10 minutes after reperfusion using a 14-gauge biopsy gun (ACECUT biopsy system, TSK Laboratory, Japan). The tissue specimen was subsequently halved and stored in 4% formalin or embedded (Tissue-Tek, Zoeterwoude, The Netherlands) and snap frozen in isopentane at -80°C. Seven days post transplantation renal true cut tissue samples were obtained and stored in 4% formalin or snap frozen in isopentane at -80°C.

Microscopic techniques

The collected tissue was paraffin embedded and cut into 4 µm thick sections. Light microscopy (20x magnification) of hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) stained sections were used to demonstrate changes in morphology. Histology was reviewed using the modified ischemia/reperfusion injury (IRI) scale (19). This scale quantitatively assesses the following items on a scale of 1-3 points (1 none, 2 medium, 3 severe): mesangial matrix expansion, brush border loss in proximal tubule, edema, tubular dilatation, tubular cell vacuolisation, necrosis, endothelial cell swelling and interstitial fibrosis. Points were added to create an IRI score (8-24 points). Biopsies of healthy pigs were used as reference. Immunohistochemical staining for von Willebrand factor was performed as follows: incubation for 30 minutes in pepsin solution 0.2%, pH 2,0 at a temperature of 37°C. Primary antibody:

polyclonal vWF 1:200 (Dako, Glostrup, Denmark), secondary antibody: GARPO 1:20 (Dako, Glostrup, Denmark). The findings were graded semiquantitatively on a scale of 1 to 4 for the following features: vWF staining in glomerular endothelium, subendothelium, mesangium and cortical vessels as previously described (20). The origin of all specimens remained blinded to the investigator during evaluation by light microscope.

Endothelial activation and inflammation

Real-Time Quantitative Taqman RT-PCR analysis of von Willebrand factor (vWF), monocyte chemotactic protein (MCP-1), E-Selectin and P-Selectin gene expression was performed to detect pro-inflammatory and/or pro-coagulatory activation, reflecting endothelial injury. All analyses were performed on tissue samples taken before and after preservation, 10 minutes after reperfusion and 7 days after transplantation. Total RNA was extracted from snap frozen tissue using TRIzol (Invitrogen, Breda, The Netherlands). Total RNA was treated with DNase I, Amp Grade (Invitrogen, Breda, The Netherlands). First-strand cDNA synthesis, RT-PCR and primer synthesis were essentially the same as described previously (21). All samples were assayed in triplicate. Results were normalized with the average value of the respective gene in control biopsies, arbitrarily set to 1.

Kidney injury biomarkers

Thiobarbituric acid reactive substances (TBARS) in urine were analyzed as an indication of increase in lipid peroxidation by reactive oxygen species (ROS) after reperfusion (22). Malondialdehyde binds to thiobarbituric acid and the subsequently formed TBARS were extracted in a butanol layer, measured with a fluorescence spectrophotometer at 485/590 nm (Baun de Ronde FL 600, Abcoude, The Netherlands). Urinary protein content was assayed via the method of Lowry and colleagues (23). Activity of brush border enzymes alanine aminopeptidase (AAP) and lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) in urine and perfusate were measured using colorimetric assays (24-26).

Statistics

Data are expressed as mean ± standard error of mean (SEM). Statistical significance of differences was assessed using the Kruskal-Wallis test followed by a Mann-Whitney test. A p-value ≤0.05 was considered to indicate statistical significance.

Results

No significant differences in cold ischemic times (CIT) and anastomosis times were observed between groups. The mean (± SEM) CIT for the CS, HMP 30/20 and HMP 60/40 groups were 20:26 ± 0:16, 20:23 ± 0:13 and 20:26 ± 0:10 h, respectively. The mean ± SEM anastomotic times were 32 ± 1, 30 ± 1, 35 ± 1 min respectively.

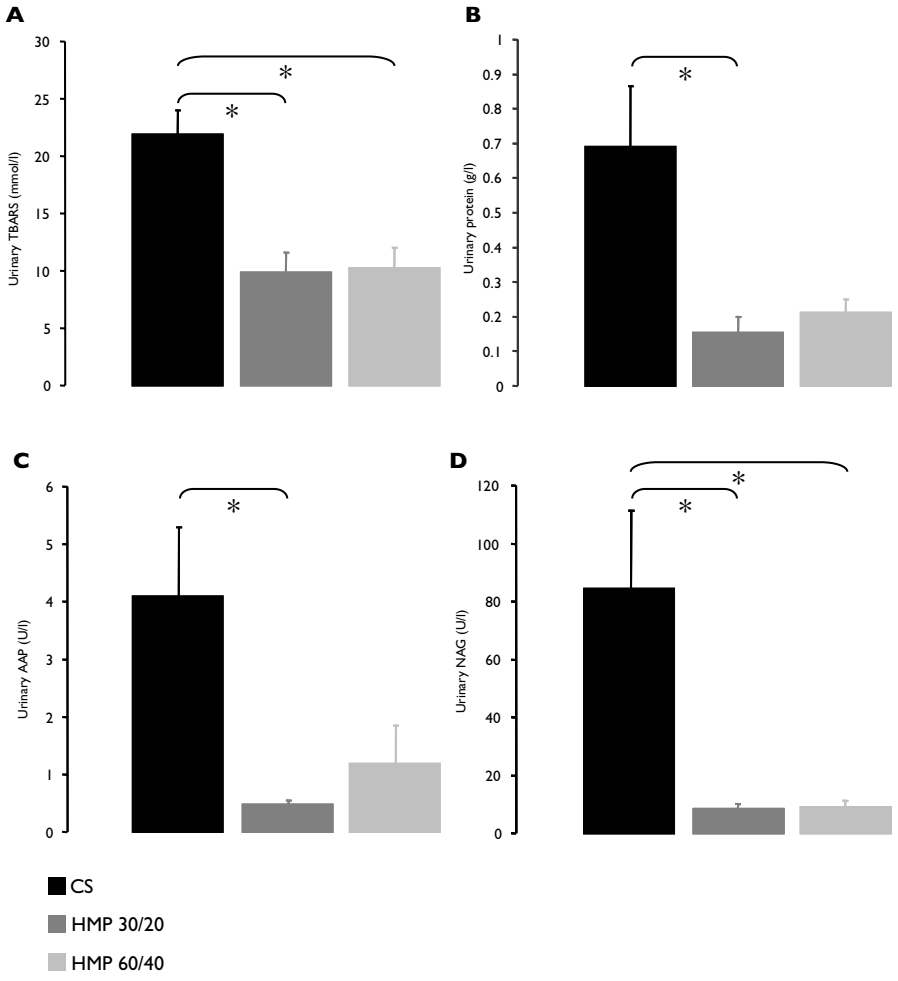

Figure 2. Urinary damage markers (mean + standard error of the mean) measured in first 2 ml of urine after reperfusion. Thiobarbituric acid-reactive substances (TBARS) **(A)**, proteinuria **(B)**, alanine-aminopeptidase (AAP) **(C)** and N-acetyl-β-D-glucosaminidase (NAG) **(D)**. * $p<0.05$.

The first warm ischemia time (time between arterial clamping and cold flush out) ranged from 2-6 minutes and did not differ significantly between groups. In both CS and HMP 30/20 groups all animals survived the seven day follow up period. In contrast, only three out of five animals survived in the HMP 60/40 group. Both deaths were due to renal transplant failure.

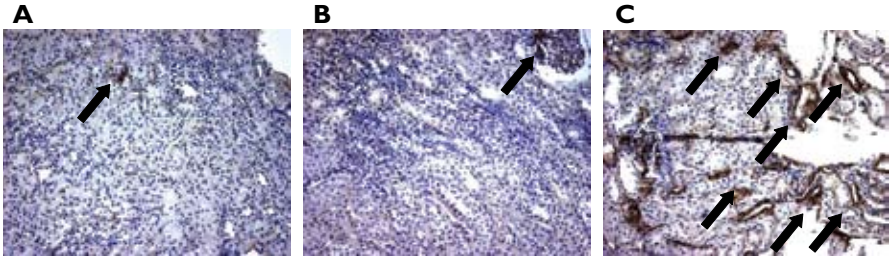

Figure 3. Representative section of kidney cortex biopsies after preservation immunostained (arrows) for von Willebrand factor (vWF) in CS **(A)**, HMP 30/20 **(B)** and HMP 60/40 **(C)** preserved grafts. Significantly more vWF staining was observed in HMP 60/40 preserved kidneys compared to CS and 30/20 ($p<0.05$).

Perfusion dynamics

Renal artery flow measurements during HMP showed a significant increase in flow, from 38 ± 2 ml/min initial to 65 ± 4ml/min after 20 h in the HMP 30/20 group. This indicates a reduction of vascular resistance over time. In the HMP 60/40 group a similar pattern was observed with an initial flow of 49 ± 7 ml/min rising to 104 ± 10 ml/min at 20 h. Continuous temperature registration showed a stabilization of graft core temperature at 2-4°C after 2 h of HMP in both groups.

Reperfusion injury

ROS formation

Measurement of TBARS in urine produced immediately after reperfusion allowed detection of ROS production. TBARS levels in urine from CS grafts were significantly higher compared to HMP 30/20 and HMP 60/40 groups. Urinary TBARS after CS were 21.9 ± 2.2 mmol/l compared to 9.8 ± 1.2 and 10.3 ± 1.8 mmol/l after, respectively, HMP 30/20 and HMP 60/40 (Fig. 2).

Proximal tubule damage

Detection of NAG and AAP activity in urine allowed an assessment of injury to the proximal tubule immediately after reperfusion. Urine concentrations of NAG were significantly higher in urine from CS grafts compared to both HMP groups. Urinary NAG concentrations were 84.2 ± 27.1 U/l after CS compared to 8.5 ± 1.7 U/l after HMP 30/20 and 8.9 ± 2.3 U/l after HMP 60/40. The results with AAP and proteinuria showed a similar pattern (Fig. 2).

Microcirculation

Microcirculatory tissue perfusion of the renal cortex 10 minutes after reperfusion showed significant differences between groups. Cortical erythrocyte flux was significantly higher in both HMP groups, averaging 130 ± 10% (HMP 30/20) and 109 ± 4% (HMP 60/40) of controls compared to 85 ± 8% in CS grafts ($p<0.05$).

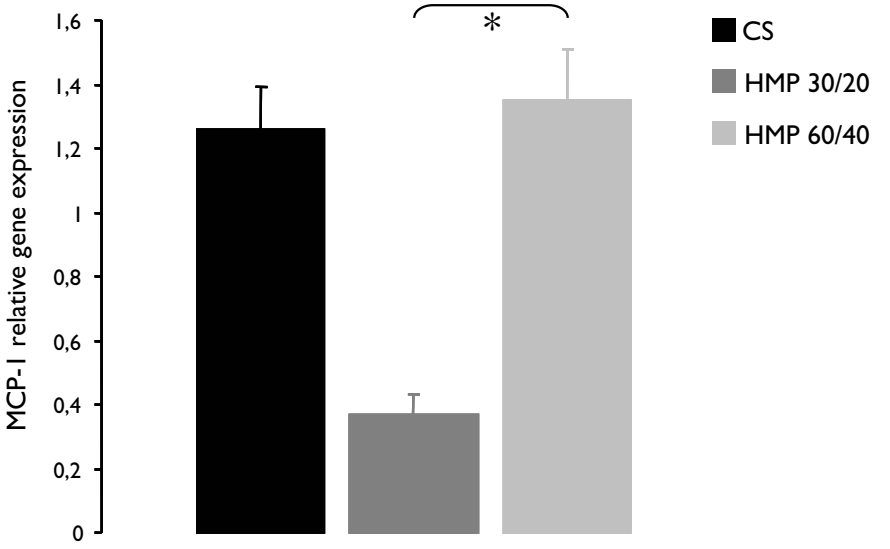

Figure 4. Monocyte chemotactic protein (MCP-1) relative gene expression in kidney cortex after preservation. A significantly lower expression of MCP-1 was seen in HMP 30/20 grafts compared to HMP 60/40 preserved kidneys. * $p<0.05$.

Renal histology

The modified ischemia/reperfusion injury (IRI) score did not differ significantly between groups. Overall, low scores (maximum 12/24) were observed. Although a moderate increase of damage was seen over time, this did not reach statistical significance.

Endothelial activation and inflammation

Three endothelial injury markers and one pro inflammatory cytokine were studied to examine the influence of CS and HMP on endothelial cell activation and inflammation. Biopsies were taken at four time points: before and after preservation, after reperfusion and at autopsy. In kidney grafts preserved with HMP 30/20 von Willebrand factor (vWF) mRNA expression in the post preservation biopsy was similar to CS. An almost two fold increase vWF mRNA expression was, however, seen in HMP 60/40 preserved kidneys compared to CS grafts ($p<0.05$). These findings were confirmed with immunohistochemical staining showing increased vWF staining in HMP 60/40 (3.75 ± 0.25) grafts compared to CS (0.8 ± 0.5) and HMP 30/20 (0.6 ± 0.25) ($p<0.05$, Fig. 3). Furthermore, HMP 30/20 preserved kidneys showed a significant lower expression of MCP-1 compared to HMP 60/40 in the post preservation biopsy ($p<0.05$, Fig. 4). In contrast to vWF and MCP-1 expression, E-Selectin and P-Selectin mRNA expression did not differ significantly between groups.

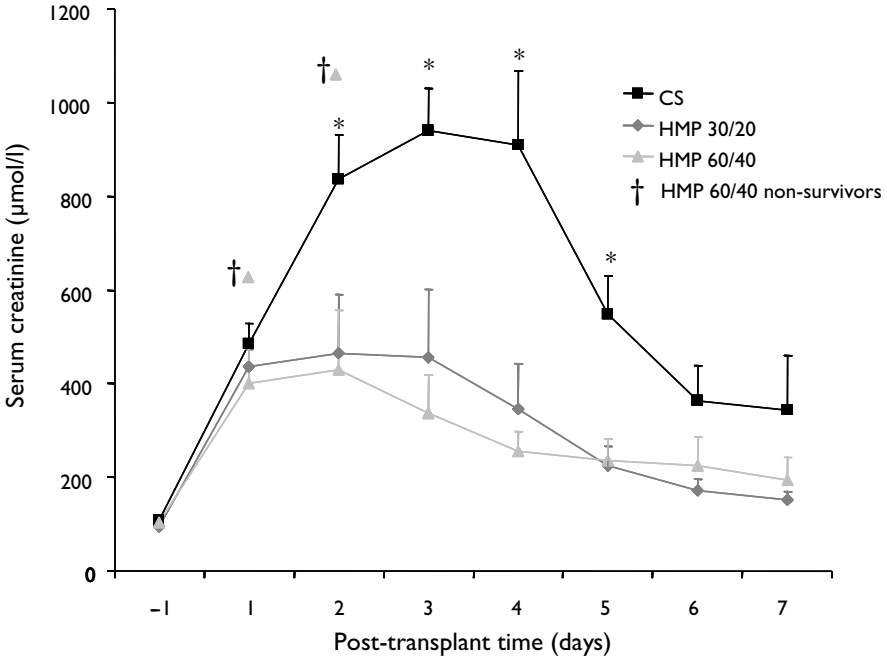

Figure 5. Post-transplant serum creatinine values in pigs after 20 h CIT with CS (n=5) or HMP 30/20 (n=5) or HMP 60/40 (n=3/5) (mean + standard error of the mean) showing lower values in HMP grafts compared to the CS group. In HMP 60/40 group only surviving animals were included. * $p<0.05$ vs. other groups.

Renal function

Post-transplant renal function data are presented in Figure 5. Preservation by HMP resulted in a significant improvement of renal function after transplantation. Peak serum creatinine levels as well as time to peak serum creatinine were significantly lower in both HMP groups. Animals with transplanted kidney grafts after CS preservation showed a peak serum creatinine of 940 ± 90 µmol/l on postoperative day 3.4 ± 0.89. In contrast, animals that received HMP 30/20 kidneys had a significantly lower peak creatinine of 463 ± 127 µmol/l on postoperative day 1.8 ± 1.1. Surviving pigs in the HMP 60/40 group showed similar results to HMP 30/20 animals with a peak creatinine of 428 ± 129 µmol/l on postoperative day 1.7 ± 0.6. However, only three animals survived with adequate renal function in this group. The autopsy on two animals with renal failure, as demonstrated by rising creatinine levels, revealed diffusely black colored grafts with open arterial and venous anastomoses (Fig. 6).

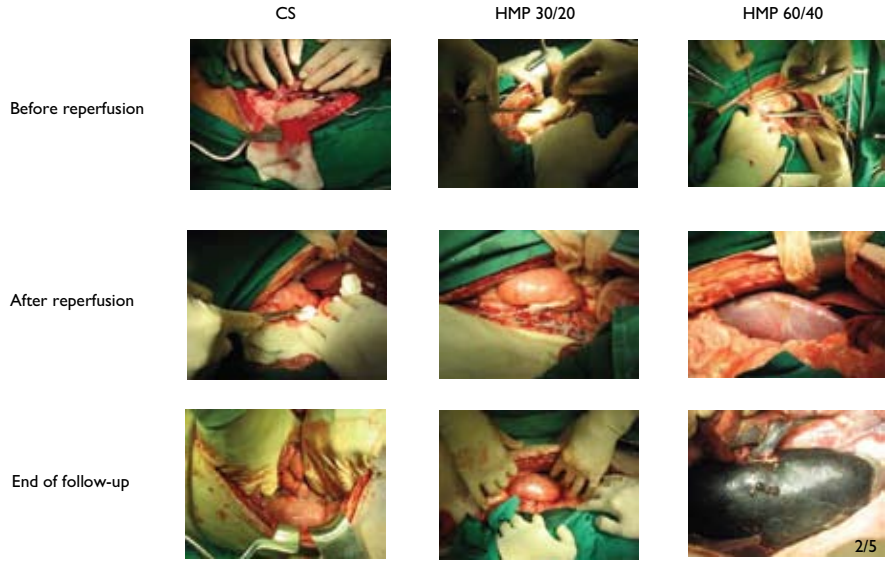

Figure 6. Macroscopic aspect of kidney grafts at different stages in the experiment. In the HMP 60/40 group a different post-reperfusion aspect was observed when compared to the CS and HMP 30/20 group. In two 60/40 cases this resulted in a thrombosed graft, without evidence of technical anastomosis faillure.

Discussion

Since many centers, due to the persistent donor shortage, will accept older, more marginal and even donation after cardiac death donors, conventional static cold storage might not be sufficient anymore to maintain organ viability during preservation. This study demonstrates that hypothermic machine perfusion at a pressure of 30/20 mmHg using the portable Groningen Machine Perfusion (GMP) system with pulsatile perfusion and oxygenation improves 20 h cold preservation of porcine kidneys. In the past, several experimental studies have addressed the role of machine perfusion in renal preservation using large animal models. Most studies available, however, have used canine kidneys, which are in contrast to man, relatively resistant to cold ischemic injury (27). The porcine kidney appears to be more sensitive to cold preservation as demonstrated by Nicholson and colleagues (1). For this reason, we have limited cold ischemia time to 20 h in our model. The pig was also chosen as an experimental model because its renal anatomy and physiology resemble the human situation (1;28;29). An autotransplant model was used to rule out allogeneic immune responses allowing unbiased investigation of ischemia/reperfusion injury. To determine renal function of the autotransplanted kidney a simultaneous contralateral nephrectomy was performed.

Cold storage preservation was chosen as a control since 80% of all kidneys is still preserved using CS (OPTN data, October 2006). The rationale for the preservation solutions used was as follows. UW-CSS and UW-MPS are quite similar solutions. UW-CSS contains lactobionate as an impermeant versus gluconate in UW-MPS and has an intracellular instead of an extracellular sodium/potassium ratio. UW-CSS is at present considered to be the 'gold standard' solution for static cold storage. In a direct comparison in the CS setting, UW-lactobionate (UW-CSS) was found to have a slightly better composition for suppression of hypothermia induced cell swelling compared to UW-gluconate (UW-MPS) (30). For this reason we used UW-CSS in the control group.

In the HMP 30/20 group, five out of five animals survived. Recovery of renal function was significantly improved compared with the CS group, with an approximate 50% reduction of peak creatinine and time to reach this maximum creatinine. As mentioned above, this improvement of post transplant viability cannot be explained by the small differences between the solutions. Compared to the study of Nicholson, values obtained in the present experiment are better, which can be explained either by the shorter CIT, i.e. 20 h versus 24 h, or the use of the GMP system instead of the Waters HMP system (1). Moreover, we could demonstrate improved microvascular tissue perfusion upon reperfusion after HMP, whereas the lower erythrocyte flux in the CS groups indicated a reduced vascular viability of these grafts. This finding is in line with previous observations that high oxygen availability during preservation improves microcirculatory parameters (4). The use of oxygen, in the GMP system, with a venous pO_2 > 100 kPa, may be especially beneficial for endothelial cells since these cells are critically sensitive to anoxia which leads to endothelial cell swelling and the so-called 'no-reflow' phenomenon. This effect may account for the impaired vascular performance of CS grafts (31-33).

Another beneficial effect of active oxygenation during preservation was seen in reduced ROS formation after reperfusion in both HMP groups. This is in accordance with earlier findings in rodent models (3;34-36). In addition to the reduced level of toxic ROS formation, less urinary release of proximal tubule injury markers AAP and NAG was observed after reperfusion of especially the HMP 30/20 preserved grafts compared to CS. Both enzymes were not detectable in UW-MPS (data not shown), thus demonstrating that the enzymes had not been already washed out during HMP. Many studies have pinpointed the damage to tubular cells as an important risk factor for the presence of delayed graft function (37). The fact that proximal tubules were less injured in the HMP 30/20 group could very well explain the earlier and better functional recovery of kidneys preserved by HMP.

A potential negative effect of HMP in general could be the occurrence of vascular injury due to shear stress (38-40). Shear stress is a parallel frictional drag force that is linearly proportional to the flow and the viscosity of the fluid (41). Obviously, shear stress can easily negatively affect the integrity of vascular endothelial cells and cause cellular detachment. In this study, pulsatile pressures of 30/20 mmHg and 60/40 mmHg were applied at 60 BPM during 20 h HMP. We could not demonstrate any significant differences between surviving animals in these groups in terms of

morphology, quantified using the modified ischemia/reperfusion injury scale. Renal function of surviving 60/40 animals was comparable to 30/20 grafts. Quantitative RT-PCR analysis and immunohistochemical staining revealed, however, a higher expression and staining of von Willebrand factor (vWF) mRNA in biopsies after preservation in the HMP 60/40 group compared to CS. The release of vWF is regarded as an important marker of endothelial cell disturbance. Perturbation of endothelial cells during HMP can indeed stimulate the synthesis of vWF (42). Platelets will subsequently adhere to vWF and initiate thrombus formation (43). This cascade might explain the observation of a more bluish aspect during reperfusion of HMP 60/40 grafts compared to CS and HMP 30/20 groups. Furthermore, the higher vWF synthesis might also explain the lower microcirculation in the HMP 60/40 group compared to HMP 30/20. More important, a marked difference in survival was seen: in the HMP 60/40 group two animals died of renal failure vs. none in the other groups. At autopsy black grafts were observed without any evidence for technical failure, e.g. arterial or venous anastomotic occlusion or complications. This observation suggests diffuse vascular damage, presumably secondary to overexpression and synthesis of vWF during HMP at 60/40 mmHg due to higher shear rates. Thus, pulsatile pressure of 60/40 mmHg using UW-MPS might damage the endothelial cells during HMP in porcine kidneys. There are some studies from the early seventies showing a relationship between perfusion pressure and endothelial damage. These studies, however, were performed with different perfusates and using rabbit or canine kidneys (39;44;45). The conclusion of these early experiments was to use a low systolic pressure of approximately 40 mmHg. Despite these studies, however, a systolic pressure of 60 mmHg is still common practice in human kidney perfusion. The present study, again, underscores the importance of perfusion pressures. Although our numbers are small and porcine kidneys are possibly more susceptible to endothelial injury, this primarily indicates that perfusion settings in human kidneys ought to be reconsidered and may be lowered.

To study the immunogenicity of the graft following preservation we measured the expression of the pro-inflammatory chemotactic cytokine MCP-1. This cytokine is important in neutrophil dependent injury and is expressed by a variety of cells including vascular endothelial cells (46). HMP 30/20 showed the lowest expression of MCP-1. Several experimental models have shown upregulation of pro-inflammatory cytokines and selectins in kidney grafts during cold ischemia (47;48). We postulate that oxygenation during preservation at low shear rates minimizes pro-inflammatory cytokine expression. Similar, in a rat liver model of oxygenated machine perfusion a marked lower expression of ICAM-1 was found in HMP grafts compared to CS (49). The higher expression of MCP-1 in the HMP 60/40 group is most probably due to shear stress which is also reflected in the higher expression and synthesis of vWF in this group.

The portable GMP uses simple but new miniature technology that will fit in the conventional organ box as currently used in transplantation. Special features include back-up with cold storage in case of total power failure and an option of normothermic perfusion by using a heat exchanger and blood compatible disposable components. The total added weight of the prototype compared to cold storage concerns only 4.8 kg.

In summary, this study demonstrates that the GMP system using pulsatile oxygenated perfusion of 30/20 mmHg at 60 BPM allows kidney preservation for 20 h in a pressure controlled manner. More important, the GMP system improved post-transplant kidney function of heart beating porcine kidneys compared to standard static cold storage with UW-CSS. Pre-set perfusion pressures, however, are found to be critically important for successful outcome after HMP. Too high perfusion pressures during HMP provoke an inflammatory response, harm the endothelium and can cause subsequent diffuse thrombosis of the graft. In contrast, if a pressure of 30/20 mmHg during HMP is applied less toxic ROS are generated, less injury to the proximal tubule occurs, less pro-inflammatory cytokines are expressed and the cortical microcirculation is improved. As we have now shown the practical feasibility of the GMP system, future studies will focus on its application in DCD donors and on a clinical comparison to other portable HMP devices.

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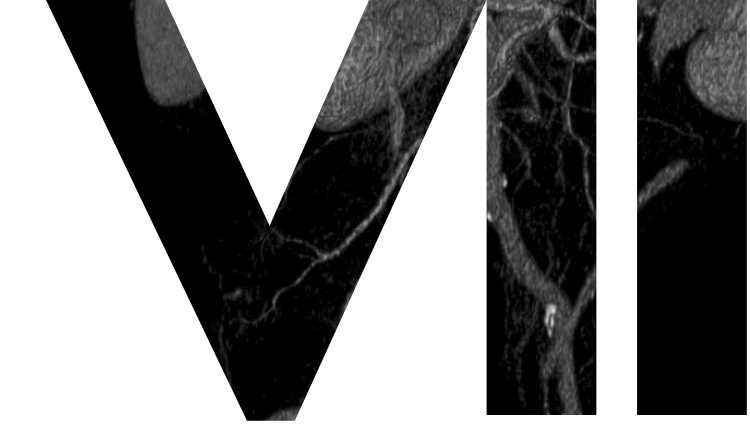

Chapter VII

Transplantation after hypothermic machine perfusion versus static cold storage of deceased donor kidneys: A prospective randomized controlled trial

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Abstract

Background: Static cold storage (CS) is the most widely used organ preservation method for deceased donor kidney grafts. Preservation by hypothermic machine perfusion (HMP) may lead to improved outcome after renal transplantation. However, there is a lack of sufficiently powered prospective studies to test the presumed superiority of HMP.

Methods: In an international prospective randomized controlled trial kidney pairs of 336 consecutive deceased donors were enrolled. One organ was randomly assigned to HMP and the contralateral kidney to CS preservation. All 672 recipients were followed up for one year. The primary endpoint was delayed graft function (DGF). Secondary endpoints were functional DGF, duration of DGF, primary non-function, serum creatinine and creatinine clearance, acute rejection, calcineurin inhibitor toxicity, length of hospital stay, and graft and patient survival post-transplant. This trial is registered as an International Standard Randomized Controlled Trial, number ISRCTN83876362.

Results: No serious adverse events directly attributable to HMP were encountered. HMP significantly reduced the risk of DGF (HMP-arm 70/336, CS-arm 89/336; adjusted OR 0.57; p=0.01), and of functional DGF. In the event that DGF occurred, its duration was shorter after HMP. Serum creatinine values were lower for HMP kidney recipients in the first two weeks post-transplant. HMP reduced the risk of graft failure in the first year after transplantation (HR 0.37; p=0.02). One year graft survival was superior in the HMP group (98% versus 94%; p=0.03). No significant differences were found for the other secondary endpoints.

Conclusion: In all common types of deceased donor kidney transplantation, HMP reduced the risk of DGF. In addition, HMP improved graft survival in the first year after transplantation.

Introduction

To date, in kidney transplantation two different organ preservation modalities are clinically used for grafts retrieved from deceased donors. In static cold storage (CS) the kidney is flushed, cooled with one of several cold preservation solutions, and transported on ice. In hypothermic machine perfusion (HMP), after an initial wash-out of blood, the kidney is connected to a perfusion device and a solution is continuously pumped through the vasculature of the organ at temperatures between 1 and 10°C (1). Compared to several decades ago, the average deceased kidney donor today is older and has been exposed to more concomitant morbidity which may have a detrimental effect on graft quality (2;3). Also, the use of organs derived from donation after cardiac death is increasing in most countries (4). Such donor grafts are known to have significantly higher rates of delayed graft function (DGF) (4;5). Evidence suggests that organs which do not function immediately after transplantation have an increased risk of acute rejection and may show worse graft survival, irrespective of the preservation solution used with static CS (6;7). In addition, DGF increases the costs of kidney transplantation (8;9). Recent retrospective studies have suggested that HMP may result in a better short-term outcome, with lower DGF rates after kidney transplantation from all types of deceased donors (9-11). Therefore, interest in HMP is increasing. In this chapter the results of the first large international prospective randomized controlled trial (RCT) comparing HMP with CS preservation in deceased donor kidney transplantation are presented. The primary endpoint is DGF.

Methods

Study design

An investigator-driven, international prospective randomized controlled trial (RCT) was designed. Donor inclusion regions were The Netherlands, Belgium and the federal state of North Rhine Westphalia in Germany. All consecutive deceased donors kidney pairs retrieved in these regions that met the initial inclusion criteria were eligible for randomization by Eurotransplant. Since the aim was to include the whole spectrum of deceased donors, no initial selection of donor types to be included was made. Thus, the study reflects the effect of HMP versus CS in everyday practice within an international organ exchange organization. A strictly paired design was chosen: from each donor, one kidney was randomized to HMP, and the contralateral organ to CS.

Follow-up data were collected from all recipients of randomized trial kidneys. Due to international sharing, these recipients were transplanted anywhere within Eurotransplant (12). Approval for the study was obtained from ethical review boards in each trial region, and from the Eurotransplant Ethical Advisory Committee and Kidney Advisory Committee.

Trial coordination and trial sponsor

An independent scientific steering committee with clinicians and scientists from each trial region was solely responsible for the design and the scientific conduct of this study. The steering committee met monthly to monitor progress and discuss any issues at hand. The study was sponsored by Organ Recovery Systems, Des Plaines, IL, USA.

Inclusion and exclusion criteria

Organ donors had to be ≥16 years of age. Only kidney pairs from deceased donors were included in the study, from either donation after brain death (DBD), or donation after cardiac death (DCD). DCD donors had to be Maastricht category III or IV (13). Due to the paired study design, kidney pairs were only included if both organs were actually transplanted into two different recipients. If one kidney was transplanted together with another organ into the same recipient, this kidney pair was excluded. The only exclusion criterion for recipients was patient death in the first week after transplantation.

Randomization

To avoid regional imbalances between the two study arms due to slightly different allocation algorithms (12), a randomization scheme based on permuted blocks within regions was used with separate randomization lists for each of the three trial regions. Randomization lists were available only to the 24 h Eurotransplant duty desk. Upon report of a kidney donor, the allocation officer first checked its eligibility and then assigned the left kidney to treatment with either HMP or CS following one of the three randomization schemes, which automatically assigned the right kidney to preservation with the other method. Then both kidneys were offered according to the match list, without revealing the preservation method. Only if the kidney assigned to be machine perfused had a too small aortic patch or too many renal arteries preventing a reliable connection to the HMP device, surgical teams were allowed to switch randomization during organ procurement.

Logistics

In each trial region a team of trained perfusionists was available 24 h per day, 7 days per week to respond to donor reports. Perfusionists transported the HMP device to the donor hospital, assisted donor surgeons with placing one kidney on the machine. No changes were made to existing Eurotransplant organ allocation rules or to transportation protocols. Both HMP and CS preserved kidneys were transported stand-alone to their respective recipient center. After transplantation, empty HMP devices were returned to the nearest of the three regional coordinating centers.

Hypothermic machine perfusion

LifePort® Kidney Transporter (Organ Recovery Systems, USA) machines were used for HMP with a pulsatile flow of University of Wisconsin machine perfusion solution (Kidney Preservation Solution-1; KPS-1®) (14) at 1-8°C. Systolic perfusion pressure was fixed at 30 mmHg. Kidneys were machine perfused from organ procurement

until transplantation. To prevent any bias in clinical decisions about transplanting or discarding an organ, HMP dynamics data -such as renal vascular resistance and flow readings- were never revealed to the transplant team. A safety board of experienced transplant surgeons was installed and consulted on three occasions: In two out of three cases it felt the need to reveal HMP dynamics data to the recipient center. In both of these cases the recipient centers saw no reason for organ discard based on the additional information and transplanted the kidney.

Cold storage

No changes were made to standard CS protocols; grafts were cold stored according to established Eurotransplant routine. After an initial vascular flush-out, kidneys were submerged in the preservation solution and stored on melting ice.

Data collection

Donor procedure-related data were collected by the perfusionist on duty. Recipient follow-up data were provided by all 60 participating transplant centers within Eurotransplant through a secure online database. A random sample of 10% of all cases was checked for accuracy by means of an external independent data audit. No relevant irregularities were found.

Endpoints

The primary endpoint was DGF, defined as dialysis requirement in the first week after transplantation. Secondary endpoints were: duration of DGF, primary non-function, area under the curve of daily serum creatinine at days 1–14, creatinine clearance at day 14, biopsy proven acute rejection, calcineurin inhibitor (CNI) toxicity, recipient length of hospital stay, and graft and patient survival up to one year post-transplant. Graft survival was censored upon patient death with a functioning graft. To filter out those graft faillures that were due to surgical-technical complications rather than the biological conduct of the graft, all graft analyses were condtioned on 14 days' graft survival (15). In addition to the primary endpoint we have also examined DGF by a more functional definition from Boom (16). Functional DGF (*f*-DGF) was adopted as secondary endpoint and defined as the absence of a decrease in serum creatinine of at least 10% per day for at least three consecutive days in the first week after transplantation, not including patients who developed acute rejection and/or calcineurin inhibitor toxicity in this first week. All endpoints mentioned above were pre-specified in the study protocol, except primary non-function, which was added post hoc.

Statistics

This study was powered to find a reduction in DGF of at least 10%, based on a presumed 35% DGF incidence in CS kidney recipients. With a power of 0.8 and a type I error of 0.05, the minimum required sample size was 300 kidney pairs. The primary analysis of the DGF endpoint consisted of a logistic regression model, which examined whether HMP versus CS, in the context of other relevant donor-,

preservation-, and recipient-related factors, influenced DGF risk (7;17). Covariates for this model (Table 2) were pre-specified in the study protocol, and their choice was based on literature. The final model was determined by entering all covariates at once in the analysis, with a built-in normal gamma frailty term for the donor to account for the paired study design (18). For endpoint variables, univariate differences between groups were assessed with the McNemar test (discrete variables), or the Wilcoxon signed rank test (continuous variables). For demographic variables, univariate differences were assessed with Fisher's exact test, or the Mann-Whitney test. The Kaplan–Meier method analyzed graft and patient survival. Differences between survival curves were determined using log-rank tests. A Cox proportional hazards model was applied to examine which variables significantly influenced the risk of graft failure. To construct this model, a similar approach as for the logistic regression model for DGF was followed: The same set of covariates was entered all at once, and a normal gamma frailty term for the donor was included to account for the within-donor dependence structure of the data.

Prespecified subgroup analyses of DCD versus DBD, and expanded criteria donation (ECD) versus standard criteria donation (SCD) were performed for the treatment effect on the primary endpoint. Interaction terms were added to the logistic regression model described above, to test whether the magnitude of the effect of HMP on DGF differed significantly between subgroups (19). ECD was defined as donor age ≥60, or donor age between 50 and 60, with at least two of the following additional donor characteristics: (a) history of hypertension, (b) cerebrovascular cause of death, (c) pre-retrieval serum creatinine >132 μmol/l (20). No other subgroup analyses were performed than those reported in this chapter.

For all analyses, a p-value ≤0.05 was considered to indicate statistical significance. All reported p-values are two-sided, and not adjusted for multiple testing. Analyses were conducted using SPSS, SAS, and R software packages and are based on all organ pairs that met the inclusion criteria.

No interim analyses of study endpoints were carried out. A confidential safety analysis was performed at regular intervals, which compared reported adverse event rates between the two trial arms.

Results

From 1 November 2005 through 31 October 2006, 654 potential deceased kidney donors aged ≥16 years in the three trial regions were reported to the Eurotransplant desk. Figure 1 shows how 336 kidney pairs (672 recipients) were finally included in the analysis. No serious adverse events directly attributable to HMP were encountered. Table 3 povides an overview of reported adverse events. In 4.6% of all cases, initial randomization was switched due to aberrant vascular anatomy of the kidney to be machine perfused. We checked whether vascular anomalies had any influence on this study's primary outcome or on the risk of graft failure. No significant effect on DGF was found.

Table 1. Demographics and univariate statistics of endpoints*

Variable	HMP-arm (n = 336)	CS-arm (n = 336)	p-value
Donor demographics			
Donor age (yr)	51 (16-81)		-
Donor type (DBD / DCD)	294 / 42		-
Donor type (SCD / ECD)	242 / 94		-
Recipient demographics			
Age (yr)	53 (11-79)	52 (2-79)	0.21
Duration of pre-transplant dialysis (yr)	4.5 (0.15-18)	4.4 (0.19-24)	0.59
Previous transplants (%)†	23	21	0.38
PRA levels (0-5% / 6-84% / >84%)	297 / 35 / 4	304 / 29 / 3	0.68
Immunosuppressive drugs (%)			
Prednisolon	98	99	0.77
Cyclosporin	50	54	0.25
Tacrolimus	49	46	0.39
Azathioprine	1	2	0.18
Mycophenolate mofetil	86	87	0.73
Anti-thymocyte globulin	14	13	0.82
Interleukin-2 receptor antagonists	42	47	0.18
Transplant demographics			
HLA mismatches (% of 0 mismatches)‡	16	15	0.90
Cold ischemic time (h)	15.0 (3.5-29.7)	15.0 (2.5-29.7)	0.30
Primary endpoint – univariate			
Delayed graft function (%)	20.8	26.5	0.05
Secondary endpoints – univariate			
f-DGF#	22.9	30.1	0.03
Primary non-function (%)	2.1	4.8	0.08
Duration of DGF (days)	10 (1-48)	13 (1-41)	0.04
Creatinine clearance at day 14 (ml/min)	42 (0-171)	40 (0-175)	0.25
CNI toxicity within 14 days (%)	6.3	5.7	0.86
Acute rejection within 14 days (%)	13.1	13.7	0.91
Post-transplant hospital stay (days)	19 (4-392)	18 (6-382)	0.78

* If not indicated otherwise, values are expressed as median (range). For endpoint variables, p-values were obtained by McNemar test for discrete variables and by the Wilcoxon signed rank test for continuous variables. For baseline characteristics, p-values were obtained by Fisher's exact test for discrete variables, and by the Mann-Whitney test for continuous variables.
† Indicates the percentage of recipients who had undergone one or more renal transplants prior to the one included in this analysis.
‡ Indicates the percentage of transplants with zero HLA A/B/DR mismatches.
# *f*-DGF was defined as the absence of a decrease in serum creatinine of at least 10% per day for at least three consecutive days in the first week after transplantation, not including patients who developed acute rejection and/or calcineurin inhibitor toxicity in this first week.

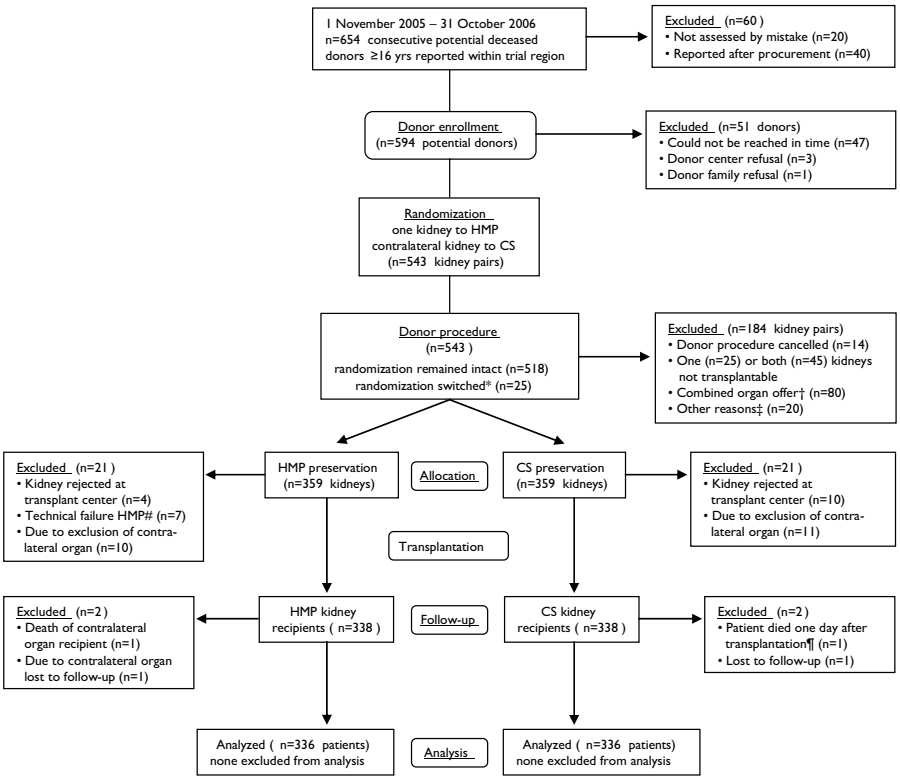


Figure 1. Enrollment, group assignment, follow-up and analysis.
* Switching randomization was only allowed when vascular anatomy made one kidney less suitable for the machine.
†Whenever one or both kidneys were offered together with another organ to one recipient, e.g. for combined pancreas–kidney or liver–kidney transplantation.
‡ Other reasons for exclusion were: Twelve of the adverse events reported in table 3, and in addition five cases in which the donor was monorenal, two cases in which consent for kidney donation was withdrawn just before procurement, and one DCD Cat. III procedure which was converted to a DCD Cat. II procedure.
# None of these failures rendered the graft unsuitable for transplantation. When HMP failed, the kidney was automatically cold stored inside the machine.
¶ Cause of death was a non-transplant related event.

Aberrant vascular anatomy did have a significant detrimental effect on graft survival (HR 2.41; p=0.03), but addition of this factor to the Cox model had no effect on the HMP versus CS hazard ratio (see also the supplementary appendix).

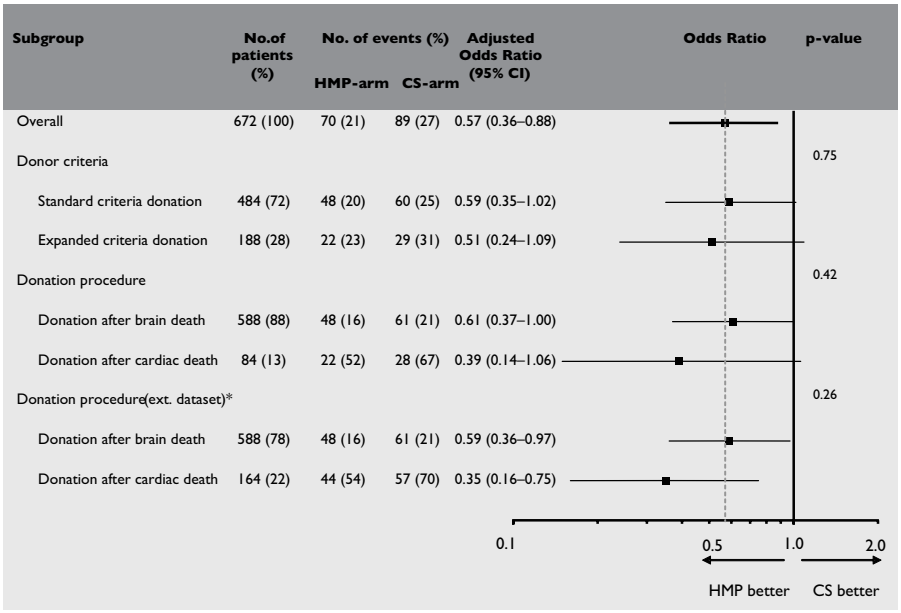


Figure 2. Subgroup analysis.
Number of DGF events per study arm, and adjusted odds ratios (black squares) with 95% CIs (horizontal lines) for DGF. P-values for the interaction between the treatment effect (HMP versus CS) and any subgroup variable. The scale of the x-axis is logarithmic. All subgroup analyses shown in this figure were pre-specified.
* Extended dataset refers to the main dataset, plus the 80 additional DCD kidney recipients who were enrolled after main inclusions had ended. This extended dataset (n = 752 recipients) was used solely to provide more statistical power for a meaningful DCD/DBD subgroup analysis.

Demographics

Table 1 summarizes characteristics of the study groups. All DCD donors were Maastricht category III. For the recipient variables age, pre-transplant dialysis duration, previous transplants, and panel-reactive antibodies (PRA), values did not significantly differ between the two arms. There were no significant differences between the two arms in HLA mismatches and cold ischemic time (CIT).

Delayed graft function

In the HMP-arm 70 recipients developed DGF, and in the CS-arm DGF occurred in 89 patients (20.8% versus 26.5%, p=0.05). Table 2 shows the logistic regression model. Compared to CS, HMP significantly reduced the DGF risk with an odds ratio (OR) of 0.57 (p=0.01). In addition, donor age (OR 1.03; p=0.04), CIT (OR 1.08; p=0.003), duration of pre-transplant dialysis (OR 1.16; p=0.01), re-transplantation versus first transplant (OR 3.01; p<0.001), and DCD versus DBD donation (OR 17.2; p<0.001) were all independent determinants of an increased risk of DGF.

Table 2. Multivariate risk analysis for delayed graft function and graft failure*

Variable	Odds Ratio / Hazard Ratio (95% CI)†	p-value
Delayed graft function		
HMP vs. CS	0.57 (0.36 – 0.88)	0.01
PRA level (%)	1.01 (0.99 – 1.02)	0.29
Recipient age (yr)	1.01 (0.99 – 1.03)	0.28
Donor age (yr)	1.03 (1.00 – 1.06)	0.04
ECD donor vs. SCD donor	1.04 (0.46 – 2.34)	0.92
Cold ischemic time (h)	1.08 (1.03 – 1.14)	0.003
Duration of pre-transplant dialysis (yr)	1.16 (1.03 – 1.31)	0.01
Number of HLA mismatches	1.13 (0.94 – 1.37)	0.18
Re-transplant vs. first transplant	3.01 (1.75 – 5.18)	<0.001
DCD donor vs. DBD donor	17.2 (8.16 – 36.2)	<0.001
Graft failure within one year post-transplant‡		
HMP vs. CS	0.37 (0.16 – 0.86)	0.02
Duration of pre-transplant dialysis (yr)	0.94 (0.78 – 1.14)	0.54
Recipient age (yr)	0.96 (0.93 – 0.99)	0.02
Donor age (yr)	1.04 (0.98 – 1.10)	0.16
Cold ischemic time (h)	1.06 (0.97 – 1.15)	0.20
PRA levels (%)	1.01 (0.99 – 1.03)	0.55
Number of HLA mismatches	1.32 (0.97 – 1.81)	0.08
DCD donor vs. DBD donor	1.50 (0.38 – 5.92)	0.56
Re-transplant vs. first transplant	1.77 (0.72 – 4.32)	0.21
ECD donor vs. SCD donor	2.44 (0.66 – 9.04)	0.18

* Logistic regression model for delayed graft function and Cox proportional hazards model for graft failure.
† Odds ratios apply to delayed graft function and hazard ratios apply to graft failure.
‡ Censored upon death with a functioning graft and conditioned on 14 days' graft survival.

Table 3. Adverse events reported in the first year after transplantation

Event	HMP-arm	CS-arm
Adverse events during donor procedure*	Number of cases (%)	
Both kidneys had vascular anatomy unsuitable for HMP†	7 (2)	
Surgical team insisted to put both kidneys on HMP†	4 (1)	
Surgical team refused to cooperate with study	3 (1)	
13 Year old donor randomized by mistake†	1 (0)	
Renal polar artery overlooked during procurement#	1 (0)	
Adverse events during organ preservation		
Technical failure / malfunction HMP‡	7 (2)	n.a.
Delayed delivery of crossmatch material¶	1 (0)	0 (0)
Serious adverse events in recipients		
Any serious event	77 (23)	88 (26)
Severe urinary tract infection	11 (3)	10 (3)
Sepsis due to any cause	9 (3)	10 (3)
Diabetes mellitus	9 (3)	10 (3)
Severe respiratory tract infection	8 (2)	14 (4)
Postoperative bleeding	8 (2)	8 (2)
Peritonitis	6 (2)	5 (2)
Any arterial thrombosis	6 (2)	4 (1)
Any venous thrombosis	6 (2)	4 (1)
Any cancer	4 (1)	9 (3)
Severe gastro-intestinal tract infection	4 (1)	5 (2)
Cardiac decompensation	3 (1)	3 (1)
Myocardial infarction	2 (1)	2 (1)
Ileus	1 (0)	3 (1)
Gastro-intestinal bleeding	0 (0)	2 (1)
Minor adverse events in recipients		
Any minor event	170 (51)	148 (44)
Uncomplicated urinary tract infection	43 (13)	47 (14)
CMV infection / reactivation	23 (7)	29 (9)

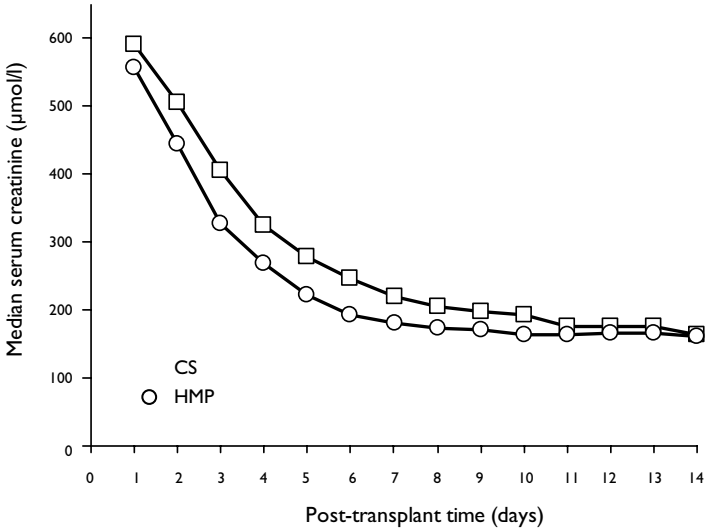
Table 3. Adverse events reported in the first year after transplantation (continued)

Event	HMP-arm	CS-arm
Minor adverse events in recipients		
Uncomplicated gastro-intestinal tract infection	22 (7)	21 (6)
Seroma	20 (6)	13 (4)
Ureteral stenosis (graft)	12 (4)	5 (2)
Anemia	11 (3)	8 (2)
Elektrolyte disturbances	9 (3)	5 (2)
Leukopenia	7 (2)	2 (1)
Wound abscess	5 (2)	3 (1)
Hydronephrosis of unknown cause (graft)	5 (2)	2 (1)
Mild cardiac arrhythmia	5 (2)	2 (1)
Incisional hernia	4 (1)	5 (2)
Upper respiratory tract infection	2 (1)	6 (2)
Renal capsular hematoma due to biopsy§	2 (1)	n.a.

All serious adverse events except study endpoints are listed in this table. No serious adverse events directly attributable to HMP were reported. Of all minor adverse events, only those that occurred in ≥1% of all cases are listed. No statistical tests were performed on data shown in this table.
* All these events led to exclusion of the kidney pair from the study (see also Fig. 1).
† Part of exclusions for "other reasons", as reported in Figure 1.
‡ None of these events rendered the graft unsuitable for transplantation. When HMP failed, the kidney was automatically cold stored inside the machine.
# One kidney was unsuitable for transplantation due to insufficient length of the remaining polar artery.
¶ Transplantation was postponed for three hours due to delayed crossmatch.
§ For an amendment to the study protocol, addressing additional research questions not reported in this chapter, cortical biopsies were taken from several machine perfused kidneys. Capsular hematomas did not compromise function of these kidneys.

Subgroup analysis

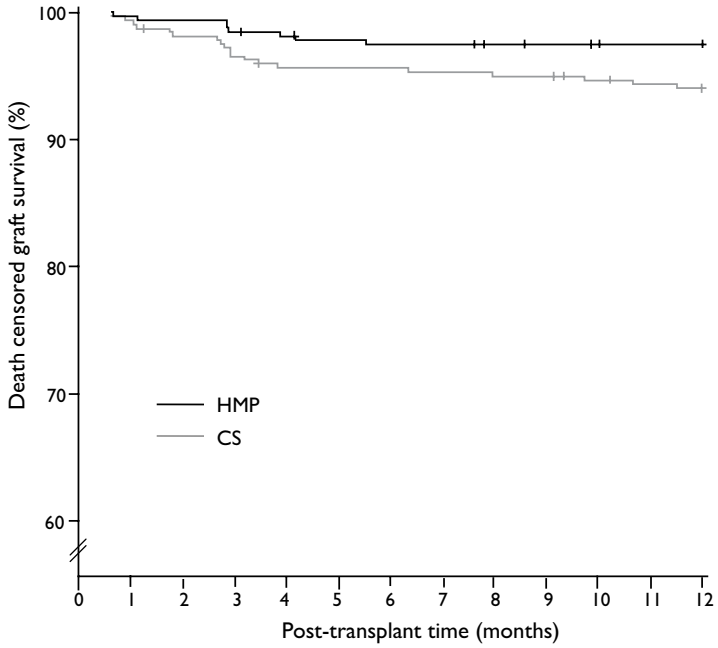
In September 2006, when donor inclusions into the study were nearly complete, the scientific steering committee expected that insufficient DCD donors would be enrolled at trial completion to conduct a meaningful subgroup analysis for this donation type. With permission of all centers, inclusions of only DCD donors were extended, until a total of 82 were enrolled on 17 August 2007. A Consort diagram and demographics for the two eras of DCD donor inclusions can be found in the supplementary appendix. Solely for the DBD/DCD subgroup analysis, these additional inclusions were added to the main set of cases to provide more statistical power. The main set of cases



No. of values available														
CS-arm	327	329	328	319	322	309	316	282	295	268	266	242	248	278
HMP-arm	322	324	325	326	316	313	314	296	286	281	273	251	245	274
Median serum creat. (µmol/l)														
CS-arm	592	507	406	327	283	248	221	210	200	195	180	180	177	166
HMP-arm	557	455	328	269	224	193	182	173	174	168	165	171	168	162

Figure 3. Time course of serum creatinine after transplantation.
Below the x-axis, the sample size available per post-transplant day, and median serum creatinine values per day are indicated. For each recipient, the area under the curve (AUC) was calculated. Missing values (12%) were imputed by means of linear interpolation. The median AUC of HMP recipients (1456; range 385–5782) was significantly lower than the median AUC of CS recipients (1787; range 288–6500) (p=0.01; Wilcoxon signed rank test).

consisted of 336 kidney pairs (672 recipients), of which 42 (84 recipients) came from DCD donors. The extended dataset comprised a total of 376 kidney pairs (752 recipients). This total includes the 80 recipients of 40 DCD kidney pairs who were later added to the main set for this analysis only. Therefore, the total number of DCD kidney pairs in the extended dataset was 82 (164 recipients). The same logistic regression model for DGF with an interaction term for HMP and DCD was applied to the extended dataset. Figure 2 shows a forest plot of the treatment effect in subgroups. First, interactions for subgroups SCD/ECD and DBD/DCD are shown, based on the main set of inclusions. No significant difference in the magnitude of the treatment effect was found for these subgroups (p=0.75 and p=0.42, respectively). When the DBD/DCD interaction test was performed on the extended dataset, there was also no significant difference between the impact of HMP versus CS in DBD versus DCD kidney grafts (p=0.26).



No. at risk													
HMP-arm	324	323	322	319	317	315	314	314	312	311	310	309	309
CS-arm	321	319	314	309	305	305	305	304	303	303	300	298	297

Figure 4. Death censored graft survival.
Both curves are conditioned on 14 days' graft survival. Below the graph, the number of recipients at risk for graft failure is indicated for each month post-transplant. Vertical lines indicate cases censored upon death with a functioning graft. HMP versus CS: p=0.03 (log-rank test).

Secondary endpoints

Table 1 also shows univariate statistics for all secondary endpoints. *f*-DGF occurred in *77* recipients in the HMP-arm and in 101 recipients in the CS-arm (22.9% versus 30.1%, p=0.03). The incidence of primary non-function in CS recipients was more than two times higher than in HMP recipients, but this difference did not reach statistical significance (4.8% versus 2.1%, p=0.08). When recipients developed DGF, the duration was three days shorter after HMP than after CS (10 days versus 13 days; p=0.04). Creatinine clearance at 14 days post-transplant, length of hospital stay of recipients, the incidence of CNI toxicity, and acute rejection rate in the first 14 days after transplantation showed no significant differences between the two study arms. The curve of daily serum creatinine evolution in the first two weeks post-transplant (Fig. 3) was significantly lower for HMP kidney recipients (p=0.01).

Patient and graft survival

At one year post-transplant, patient survival was 97% for both, HMP and CS kidney recipients. One year graft survival (Fig. 4) in the HMP-arm was significantly higher than in the CS-arm (98% versus 94%, p=0.03). In the Cox regression analysis (Table 2), HMP significantly reduced the risk of graft failure in the first year post-transplant with a hazard ratio of 0.37 (p=0.02). To test the hypothesis that this beneficial effect of HMP on the risk of graft failure could be mediated through a reduction in DGF, a post hoc analysis was conducted: DGF was added as a time dependent covariate to the Cox model. DGF proved to be a significant determinant of an increased risk of graft failure (HR 1.63; p<0.001), the hazard ratio of HMP versus CS increased to 0.66, and this covariate became non-significant in the model (p=0.15) (see also the supplementary appendix).

Discussion

Static cold storage is the easiest and currently most widely used preservation method in kidney transplantation (USA: 80%, Eurotransplant: ~100%) (21;22). Although recent retrospective studies have suggested HMP superiority (9;10), these registry analyses are biased due to selection of donor kidneys to be pumped and organ discard based on perfusion parameters (23). To date, no reliable prospective RCTs have been conducted to test the presumed superiority of HMP. Only a limited number of prospective studies have been performed, unfortunately either without adequate randomization or with equivocal results due to small sample sizes (24-28).

This study is the first large, prospective RCT to show that HMP significantly reduces the risk of DGF. Due to its size and strictly paired design, the study was probably better capable of detecting even modest differences in outcome between HMP and CS preservation. The rather large number of exclusions is typical for a paired study in organ preservation: Randomization inevitably had to be carried out at a very early stage in the donation cascade, when there was merely a potential kidney donor. Only after both kidneys had actually been transplanted could it be determined whether a donor would meet the inclusion criteria. Exclusion of donors of whom one kidney was discarded may have led to a mild bias toward the "better" kidney donor in our study. The same might be true for donors who were not included because the donor hospital could not be reached in time by the perfusionist: Theoretically, these donors may have been more unstable ICU patients, in whom organ procurement had to be performed as a quick emergency procedure. Conversely, excluding donors from whom combined kidney-pancreas transplants were performed may have slightly biased the data in the opposite direction, as in general only the most optimal donors are considered for these procedures. In a small number of cases initial randomization was switched for anatomical reasons. It is unlikely that this practice has significantly biased the study's outcomes: Aberrant vascular anatomy did not have a significant effect on DGF, and although it did influence graft survival, addition of this factor to

the Cox model did not cause a relevant change in the observed effect of the HMP versus CS covariate. In the design of this study, we chose to employ a multivariate model as the primary analysis of DGF, because of the anticipated heterogeneity which is common for transplant recipient populations (22;29).

The assumed 10% reduction we initially powered for (35% to 25% = OR 0.62) was not demonstrated in a univariate analysis (26.6% to 20.8% = OR 0.73). However, when in the logistic regression model relevant confounding factors were taken into account, the effect of HMP versus CS on DGF risk had an OR under 0.62. The effect found is slightly stronger than the associations observed in major retrospective studies and meta-analyses (OR 0.62-0.73) (9;10). Median CIT in both treatment arms (15.0 h) was relatively short when compared to other data-sets (22). This may explain why the incidence of DGF in the CS-arm of this study was 8.5% lower than the originally anticipated 35%. In addition, it is conceivable that the effect of HMP would have been stronger if cold ischemic times had been longer (22). Of interest is that HMP reduced the incidence of *f*-DGF in a more pronounced fashion (30.1% versus 22.9%) than found for the primary endpoint. Hence, the magnitude of the beneficial short term effect of HMP appears to depend to some extent on how DGF is defined.

The impact of HMP on DGF did not differ between subgroups of deceased donors. It can be argued that failure to detect a significant interaction does not imply the absence of subgroup differences. However, based on the evidence coming from the present and other studies (11), it is probably most legitimate to assume that the impact of HMP versus CS on DGF in various subgroups is at or near overall OR of 0.57. With this assumption, HMP can be considered to have a beneficial effect on short term outcome in all common types of deceased donor kidney transplantation. Nevertheless, DGF has a higher incidence in DCD and ECD kidney recipients (30). Hence, the absolute number of patients that will actually benefit from HMP could be larger in these subgroups. DCD donors in our study were solely controlled Maastricht category III donors, the effect of HMP might be more pronounced in uncontrolled, category I, II or IV DCD donor grafts that have suffered from more warm ischemic injury. Unfortunately, multicenter series of such donor categories are rare and therefore difficult to study prospectively.

Even when early graft failures are not considered, HMP has a significant protective effect against graft loss, which becomes apparent within one year after transplantation. The hazard ratio of 0.37 found in this trial indicates a remarkably stronger effect than the "mild benefit" Schold and collaegues have reported in their large retrospective study (10). The post hoc addition of DGF as a covariate to the Cox model shows that DGF renders a kidney recipient more at risk for graft failure within the first year post-transplant. In addition, it caused an increase in the HMP versus CS hazard ratio, and this covariate became non-significant in the model. Therefore, we conclude that the beneficial effect of HMP on graft survival could in part be explained by the reduction in DGF that is also caused by HMP.

The number of primary non-function cases was half as high in the HMP versus the CS group, but the difference was not statistically significant. This may be explained by

the low overall incidence of primary non-function. Although no definitive conclusions can be drawn from this observation, the phenomenon deserves close attention in future analyses. In this trial, HMP characteristics were not allowed to be used as a diagnostic tool. If they are relevant, it can be expected that such an extra selection opportunity will further increase the effect of HMP on transplant outcome due to discard of organs diagnosed to be at risk for poor outcome(23).

In conclusion, this prospective RCT comparing two preservation modalities for kidney transplantation from deceased donors found that HMP reduces the incidence of delayed graft function in all common types of deceased donor kidneys. In addition, HMP reduces the duration of DGF, in case it occurs. Machine perfused renal grafts had a lower risk of graft failure in the first year post-transplant and, as a result, these kidneys showed an improved one year graft survival versus those preserved by static cold storage.

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Supplementary Appendix

List of participating transplant centers (centers and names)

Austria (4 centers, 17 recipients) – Landeskrankenhaus Graz: H. Holzer, I. Spiegl, V. Trunk; Universitätsklinik für Chirurgie Innsbruck: R. Margreiter, C. Bösmüller, W. Mark, H. Fetz; Allgemeines Krankenhaus der Stadt Linz: B. Schmekal; Allgemeines Krankenhaus Wien: F. Mühlbacher, I. Kristo, M. Pones, G. Györi.

Belgium (7 centers, 183 recipients) – Universitair Ziekenhuis Antwerpen: D. Ysebaert, J.L. Bosmans, G. Van Beeumen, W. Van Donink; Universitair Ziekenhuis Brussel: J. Lamote, J. Sennesael, B. Amerijckx; Hôpital Erasme Bruxelles: A.D. Hoang, D. Mikhalski, D. Abramovicz, V. Brulein; Universitair Ziekenhuis Gent: C. Randon, P. Peeters, M. VanderVennet; Cliniques Universitaires St. Luc Bruxelles: M. Mourad, M. de Meyer, J. Malaise, L. De Pauw; Centre Hospitalier Universitaire Liège: J.P. Squifflet, L. Weekers, O. Detry, M.H. Delbouille; Universitaire Ziekenhuizen Leuven: J. Pirenne, Y. Vanrenterghem, F. van Gelder, B. Desschans.

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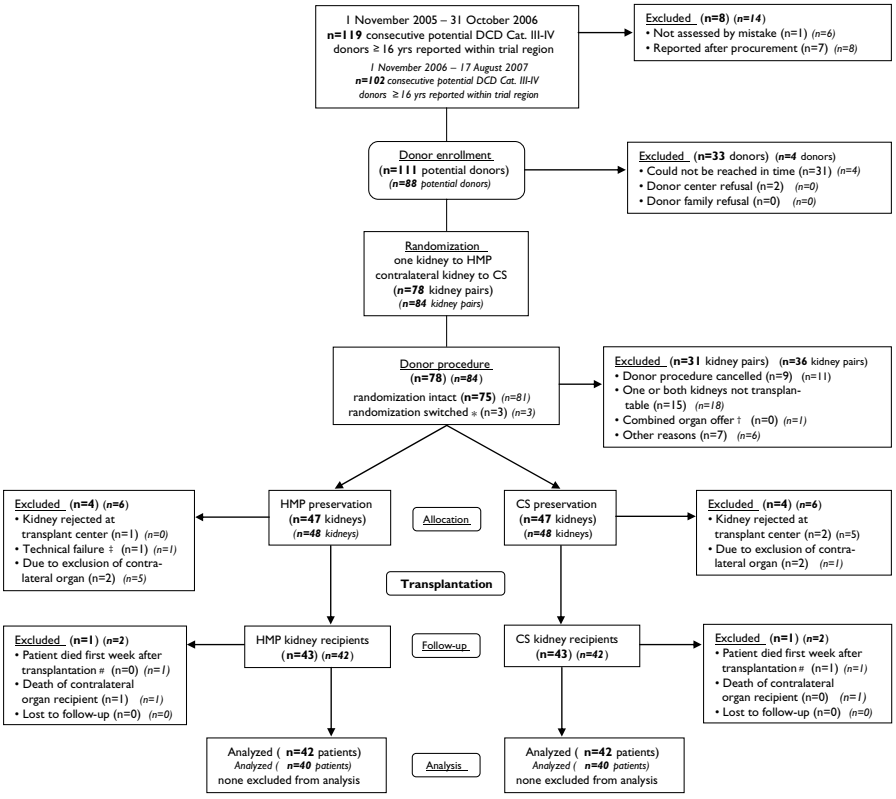

Figure S1. Enrollment, group assignment, follow-up and analysis of DCD inclusions.
Text and numbers in *italics* refer to the second era of DCD inclusions, after main consecutive inclusions (DBD and DCD mixed) had ended. These extra DCD cases were solely used for a subgroup analysis of the effect size of HMP versus CS in DCD versus DBD kidney grafts.
* Switching randomization was only allowed when vascular anatomy made one kidney less suitable for the machine.
† Whenever one or both kidneys were offered together with another organ to one recipient, e.g. for combined pancreas–kidney or liver–kidney transplantation.
‡ None of these failures rendered the graft unsuitable for transplantation. When HMP failed, the kidney was automatically cold stored inside the machine.
# Causes of death were one transplant related and two non-transplant related events.

Table S2. Demographics of both eras of DCD inclusions*

Variable	Era 1 (1 November 2005 – 31 October 2006)			Era 2 (1 November 2006 – 17 August 2007)			p-value (Era 1 vs. Era 2)
	HMP-arm (n = 42)	CS-arm (n = 42)	p-value (HMP vs. CS)	HMP-arm (n = 40)	CS-arm (n = 40)	p-value (HMP vs. CS)	
Donor demographics							
Donor age (yr)	41 (17–60)		-	48 (20–67)		-	<0.001
DCD Maastricht category (III / IV)	42 / 0		-	40 / 0		-	-
SCD / ECD	39 / 3		-	29 / 11		-	0.02
Recipient demographics							
Recipient age (yr)	49 (24–69)	51 (27–77)	0.66	48 (27–73)	53 (24–76)	0.94	0.45
Duration of pre-transplant dialysis (yr)	4.7 (1.1–18)	4.4 (1.1–11)	0.30	3.7 (1.0–10)	3.5 (0.36–8.8)	0.74	0.002
Previous transplants (%)†	19	19	0.61	0	0	-	-
PRA levels (0–5% / 6–84% / >84%)	90 / 10 / 0	81 / 17 / 2	0.17	83 / 17 / 0	93 / 7 / 0	0.16	0.46
Prednisolon (%)	98	98	-	95	100	0.25	0.67
Cyclosporin (%)	50	50	-	38	25	0.17	0.02
Tacrolimus (%)	50	52	0.50	53	75	0.03	0.12
Azathioprine (%)	2	2	-	0	0	-	-
Mycophenolate mofetil (%)	88	86	0.53	93	83	0.25	0.55
Anti-thymocyte globulin (%)	10	10	-	20	20	-	0.08
Interleukin-2 receptor antagonists (%)	31	40	0.25	45	25	0.05	0.53
Transplant demographics							
HLA mismatches (% of 0 mismatches)‡	2	0	0.10	3	8	0.83	0.31
Cold ischemic time (h)	16 (4–25)	16 (10–23)	0.54	15 (10–29)	18 (9–47)	0.41	0.70

* If not indicated otherwise, values are expressed as median (range). p-values were obtained by Fisher's exact test for discrete variables and by the Mann–Whitney test for continuous variables.

† Indicates the percentage of recipients who had undergone one or more renal transplants prior to the one included in this analysis.

‡ Indicates the percentage of transplants with zero HLA A/B/DR mismatches.

Table S3. Multivariate risk analysis for delayed graft function*

Variable	Hazard Ratio (95% CI)	p-value
Delayed Graft Function		
HMP vs. CS	0.57 (0.36–0.88)	0.01
PRA level (%)	1.01 (0.99–1.02)	0.29
Recipient age (yr)	1.01 (0.99–1.03)	0.28
Aberrant vascular anatomy†	1.02 (0.62–1.66)	0.95
Donor age (yr)	1.03 (1.00–1.06)	0.04
ECD donor vs. SCD donor	1.04 (0.46–2.36)	0.92
Cold ischemic time (h)	1.08 (1.03–1.14)	0.003
Number of HLA mismatches	1.13 (0.94–1.37)	0.18
Duration of pre-transplant dialysis (yr)	1.16 (1.03–1.31)	0.01
Re-transplant vs. first transplant	3.02 (1.75–5.22)	<0.001
DCD donor vs. DBD donor	17.2 (8.17–36.6)	<0.001

* Logistic regression model for the risk of DGF, with aberrant vascular anatomy as extra covariate, added post hoc.
† Aberrant vascular anatomy was defined as >1 renal artery of the kidney graft.

Table S4. Multivariate risk analysis for graft failure*

Variable	Hazard Ratio (95% CI)	p-value
Graft Failure within one year post-transplant†		
HMP vs. CS	0.39 (0.17–0.91)	0.03
Duration of pre-transplant dialysis (yr)	0.95 (0.79–1.14)	0.57
Recipient age (yr)	0.96 (0.93–0.99)	0.02
PRA level (%)	1.01 (0.99–1.03)	0.60
Donor age (yr)	1.03 (0.98–1.09)	0.22
Cold ischemic time (h)	1.06 (0.98–1.15)	0.17
Number of HLA mismatches	1.35 (1.00–1.83)	0.05
DCD donor vs. DBD donor	1.40 (0.37–5.29)	0.62
Re-transplant vs. first transplant	2.05 (0.85–4.97)	0.11
Aberrant vascular anatomy‡	2.41 (1.07–5.45)	0.03
ECD donor vs. SCD donor	2.77 (0.78–9.80)	0.11

* Cox proportional hazards model for the risk of graft failure within one year post-transplant, with aberrant vascular anatomy as extra covariate, added post hoc.
† Censored upon death with a functioning graft and conditioned on 14 days' graft survival.
‡ Aberrant vascular anatomy was defined as >1 renal artery of the kidney graft.

Table S5. Multivariate risk analysis for graft failure*

Variable	Hazard Ratio (95% CI)	p-value
Graft Failure within One Year Post-transplant†		
DCD donor vs. DBD donor	0.64 (0.22–1.80)	0.39
HMP vs. CS	0.66 (0.37–1.16)	0.15
Duration of pre-transplant dialysis (yr)	0.94 (0.82–1.07)	0.33
Recipient age (yr)	0.97 (0.95–0.99)	0.01
PRA level (%)	1.00 (0.99–1.02)	0.89
Cold ischemic time (h)	1.01 (0.95–1.08)	0.68
Re-transplant vs. first transplant	1.03 (0.53–2.02)	0.92
Donor age (yr)	1.03 (1.00–1.07)	0.07
Number of HLA mismatches	1.19 (0.96–1.48)	0.10
ECD donor vs. SCD donor	1.34 (0.54–3.35)	0.52
Delayed Graft Function‡	1.63 (1.29–2.05)	<0.001

* Cox non-proportional hazards model for the risk of graft failure within one year post-transplant, with DGF as extra covariate, added post hoc.
† Censored upon death with a functioning graft.
‡ DGF was added to the model as a time dependent covariate.

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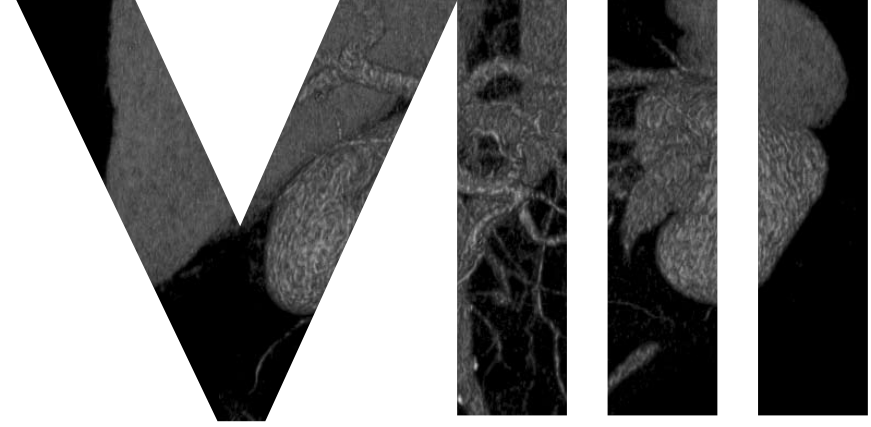

Chapter VIII

Towards normothermic preservation: effects of hemodilution on endothelial activation in vital organs

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Abstract

Background: Preservation techniques based on hypothermia such as static cold storage (CS) or hypothermic machine perfusion (HMP) have traditionally dominated organ preservation. The increase of older, marginal and ischemically damaged organs, however, has redirected attention towards novel techniques that do not induce hypothermia related damage. Normothermic preservation (NMP) provides oxygen delivery at physiologic or near-physiologic temperature and allows near-normal metabolism. Since traditional hypothermic preservation solutions cannot provide physiologic metabolic demands, diluted donor blood is often used as a preservation solution in experimental and clinical normothermic machine perfusion (NMP). In this study the effects of diluted blood were studied in a porcine hemodilution model.

Methods: Acute isovolemic hemodilution (IHD; 30 ml/kg exchange-transfusion with colloid-solutions) was induced in 15 pigs. Hypoxic oxidative stress (plasma malondialdehyde, ex-vivo oxygen radicals production in heart, lung, kidney, liver and ileum tissue biopsies), erythrocyte aggregation (LORCA), and endothelial activation (Real-Time quantitative RT-PCR on von Willebrand Factor (vWF), E- and P-Selectins, endothelial nitric oxide synthase gene-expression in tissue biopsies) were investigated.

Results: The production of superoxide and hydroxyl radicals, measured as H_2O_2 generation, was similar at all times in sham-operated and hemodiluted animals, proving a maintained oxygen delivery to tissues. Acute IHD was followed by a dramatic drop in erythrocyte aggregation and immediate pro-thrombotic (significant vWF mRNA up-regulation in heart, lung, kidney, liver and ileum) and pro-inflammatory (significant E- and P-Selectins mRNA up-regulation in lung and ileum) endothelial activation. Low erythrocyte aggregation was significantly correlated with increased mRNA-expression of vWF (heart, liver and ileum) and P-Selectin (lung, ileum and heart).

Conclusion: The results suggest that low erythrocyte aggregation during hemodilution triggers endothelium-dependent thrombogenic and pro-inflammatory responses. Given the potential impact of inflammation and coagulation upon reperfusion of a normothermic preserved graft the use of diluted donor blood as a normothermic preservation solution should be considered suboptimal.

Introduction

Organ preservation has always been crucial for transplant outcome, but will become even more important in the present era with increasing numbers of older, more marginal and organs derived from donation after cardiac death (DCD) donors. Although static cold storage (CS) has proven its efficacy in the past, it seems that the limitations of this technique have been reached. To maintain organ viability, more efforts are necessary to reduce ischemia/reperfusion injury and initiate repair. Hypothermic machine perfusion (HMP) might be able to improve organ preservation, but suffers from the negative side effects of hypothermia such as acidosis, cell swelling, and formation of radical oxygen species. Ideally, organ preservation should facilitate the use of marginal, older and DCD organs, provide real-time viability assessment before transplantation without causing damage to the graft. Normothermic (37°C) or sub-normothermic (25-32°C) machine perfusion (NMP) is a preservation alternative that may indeed achieve these goals (1). Preservation at (near) normothermic temperatures provides more adequate ways to test and optimize graft viability and allows elimination of hypothermia induced injury (2;3). Normothermic perfusion of abdominal organs using a cardiopulmonary bypass (CPB) system followed by CS has already been applied in human kidney transplantation. This so called normothermic recirculation protocol showed significant improvements in a group of 44 DCD kidneys. PNF and DGF rates were 0% and 12.5%, respectively, compared to 22.5% and 55% for conventional preservation techniques. Despite the fact that this study was retrospective and included patients over a 12 year period, it suggests a potential benefit for clinical application of normothermic techniques (4). In experimental liver preservation, normothermic perfusion of porcine livers subjected to 60 min of warm ischemia resulted in functioning liver grafts, whereas the animals transplanted with CS livers all died. Normothermic perfused livers demonstrated stable metabolic function with adequate production of coagulation factors, hyaluronic acid clearance, glucose metabolism and significantly lower transaminases compared to CS grafts (1;5;6).

Most groups use diluted whole (donor) blood as a preservation solution during NMP. Donor blood is easy to obtain, facilitates oxygen and nutrient delivery and is fully compatible with the preserved organ. From clinical observations in CPB patients, however, it is known that perfusion with diluted blood can cause endothelial activation. Hemodilution decreases red blood cell (RBC) aggregation and plasma viscosity and thereby modifies rheological variables resulting in a constant shear stress and mechanical endothelial activation (7). The pathogenicity of hemodilution could have major implications for the use of diluted blood as an organ preservation solution. Therefore, before testing the feasibility of NMP using the Groningen Machine Perfusion (GMP) system this study examines the effects of diluted blood with a special focus on endothelial activation.

Animals, materials and methods

Animals

Fifteen female Landrace pigs, weighing 60 to 80 kg, were used in this study. All experimental procedures were approved by the Animal Experiments Committee of the University of Groningen. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed.

Experimental design

The experimental design was based on previous studies showing different effects of different molecular weights of hydroxyethyl starches (HES) on human RBCs, showing more RBC aggregation with increasing molecular weights of the colloid (8;9). Therefore, two commonly used clinical plasma expanders with different HES weights were used to induce acute isovolemic hemodilution (IHD).
The following groups were studied:

- Group 1: 30 ml/kg isovolemic exchange transfusion with HAES-sterile® 3% (HES 200/0.5, median molecular weight 200 kDa, supplemented with lactated Ringers to a final concentration of 3%, n=6);
- Group 2: 30 ml/kg isovolemic exchange transfusion with Voluven® 3% (6% HES 130/0.4, median molecular weight 130 kDa, supplemented with lactated Ringers to a final concentration of 3%, n=6);
- Group 3: control group sham-operated animals (n=3).

Anesthesia was induced with ketamine (i.m. 10 mg/kg) and diazepam (i.m. 1 mg/kg). Before intubation, ventilation was performed using a mixture of O_2 and isoflurane 4%. After tracheal intubation, ventilation was performed with isoflurane 1.5-2%.

Isovolemic hemodilution was induced after cannulation of the jugular vein and the carotid artery, by infusing HES at the arterial site, and a simultaneous venous blood withdrawal of an equal volume of blood. Hematocrit (Hct) and hemoglobin (Hb) were monitored throughout the experiment, and adjusted to a constant value of 40% of the initial value (Fig.1). No inotropic support was used. After 3 h of maintaining isovolemic hemodilution, tissue biopsies were obtained from the small intestine (ileum, luminal site), a randomly selected kidney (cortex), liver, lung and heart. Biopsies were snap frozen in liquid nitrogen and stored at -80°C to study organ-specific endothelial activation. Blood samples were collected at three time points: baseline (5 min after placement of the cannulas), post-infusion (5 min after induction of isovolemic hemodilution), and at the end of the experiment (3 h of isovolemic hemodilution).

Test of RBC aggregation

RBC aggregation measurements were performed on fresh arterial blood samples, using a Laser-assisted Optical Rotation Cell Analyzer (LORCA, R&R Mechatronics, Hoorn, The Netherlands), and quantified as Aggregation Index (AI) (10). For the determination of red cell aggregation, the blood was brought under a shear rate

of 500 s^{-1}, after which shear was stopped at t=0. The backscattered intensity of the blood layer was measured during 120 s after shear stop. The intensity dropped because of red blood cell aggregation (11).

Viscosity measurements

Viscosity measurements of plasma samples were performed with an automated dynamic shear rheometer with cone-plane geometry (AR1000 Rheometer, TA Instruments, New Castle, USA). During measurements temperature was set at 37°C and shear rate of operation at 100 s^{-1}.

Hypoxic oxidative stress

Plasma malondialdehyde (MDA)

Enzymatic detection, according to the method described by Esterbauer and Cheeseman was performed (12).

H_2O_2 production in tissue biopsies

A fluorophore-nitroxide (Molecular Probes, Eugene, USA) was used to image ex-vivo superoxide and hydroxyl radicals generated by cells (13). The reaction of fluorophore-nitroxide with superoxide results in loss of electron spin resonance signal intensity concurrent with an increase in fluorescence emission. Fluorophore-nitroxide also reacts with methyl radicals generated by the reaction of hydroxyl radicals with dimethyl sulfoxide (DMSO) (14).

Biopsies from tissue of approximately 2 mm^3 and dry weight of 2-5 mg were incubated for 10 min in a microtiter plate in 50 µl of 0.1M Tris-HCl buffer (pH 8.0), containing 2.5 mM pyruvate and 5 mM succinate to stimulate mitochondrial activity (15). Then 50 µl Tris-buffer containing 2 µM fluorescamine and DMSO (final concentration 2.5%) was added. The reaction was started after the addition of 5 µl FeII-EDTA (final Fe concentration 2 µM) in Tris buffer. In this way, both superoxide and hydroxyl radicals were converted and measured as H_2O_2 (16). The biopsies were incubated in this mixture for 10 min at room temperature. After removal of the biopsies the fluorescence was measured in a multilabel counter (Victor2, EG&G Wallac, Turku, Finland) by using 390 nm excitation and 510 nm emission filters. Standard curves were obtained by adding known amounts of H_2O_2 to the assay medium.

During incubation Hb was released from the biopsies, resulting in quenching of the fluorescence signal. Thus, a separate standard curve was prepared including stepwise diluted Hb ranging from 0.1 to 1.2 g/l. The linear relationship between Hb concentrations and fluorescence signal was used to correct for the Hb signal quenching. Hb concentration in the supernatant of the incubated biopsies was measured by the method of Harboe (17). Finally, measured H_2O_2 concentration was corrected for the dry weight of the biopsy.

Diaminobenzidine (DAB) staining

The production of H_2O_2 by cells in paraformaldehyde-fixed sections of ileum mucosa was histochemically demonstrated by incubating them for 30 min with 25

mg DAB/50 ml Tris/HCL pH 7.6, at 60°C. Catalase (150 µg/ml, 1400 U/ml) inhibited the reaction, indicating that H_2O_2 was required to produce the chromogenic DAB staining.

Endothelial activation

Real-Time quantitative Taqman RT-PCR on von Willebrand factor (vWF), E- Selectin, P-Selectin, and endothelial nitric oxide synthase (eNOS) gene expression in heart, lung, kidney, liver and intestinal tissue biopsies. Total RNA was extracted using RNeasy Mini Kits (Qiagen, Venlo, The Netherlands), as recommended by the supplier. Total RNA was treated with 2 U of DNase I (RNase-Free DNase, Qiagen, Venlo, The Netherlands) in a volume of 15 µl to remove contaminating DNA (15 min at 37°C). First-strand cDNA synthesis: the mix of RNA (1 µg), 0.25 µg random hexamer primers and 2 ng of dNTPs (Promega, Leiden, The Netherlands) was heated for 5 min at 65°C and incubated on ice for at least 1 min; subsequently. The master mix (200 U SuperScript III, Invitrogen, Breda, The Netherlands) with 4 µl of 5x first-strand buffer, 1 µl 0.1M dithiothreitol, and 40 U RNaseOUT ribonuclease inhibitor (Invitrogen) was added to the samples in a total volume of 20µl; finally a reverse transcriptase program was performed (5 min at 25°C, 60 min at 50°C, 15 min at 70°C, 4°C).

Quantitative PCR amplifications were performed on an ABI Prism 7900HT Sequence Detection System (Applera Nederland, Nieuwekerk a/d IJssel, The Netherlands). Primers and probes for von Willebrand factor, E and P-Selectin, eNOS, CD31 (endothelial marker) and GAPDH (house keeping gene) were developed commercially (Custom TaqMan Assays, Applied Biosystems-Applera Nederland BV, Nieuwekerk a/d IJssel, The Netherlands). The mRNA coordinates for the exon-exon boundaries were determined by aligning the human genomic sequences with pig mRNA sequences (Spidey alignment program, http://www.ncbi.nlm.nih.gov). As a precaution to prevent amplification of genomic DNA, primer/probe sequences were chosen such that they span exon junctions or lie in distant exons separated by long introns. The PCR step contained 1 µl of the appropriate RT reaction, 10 µl of TaqMan universal PCR master mix (Applied Biosystems), 200 nM primers, and 100 nM TaqMan probe in a final volume of 20 µl. The PCR cycling conditions were 2 min at 50°C, 10 min at 95°C, and 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. All samples were assayed in triplicate.

Relative quantification of the mRNA levels was done by subtracting the GAPDH C_T (threshold cycle) from the investigated gene C_T value ($\Delta C_T = C_{T\ gene} - C_{T\ GAPDH}$). Results were normalized with the average value of the respective gene in control sham-operated animals, arbitrarily set at 1. Results were finally expressed as $2^{-\Delta CTgene} / 2^{-\Delta CT\ CD31}$ which represents an index of the relative amount of mRNA expressed in each tissue, corrected for the amount of endothelial cells presented in each biopsy. Plasma concentrations of endothelial vWF were investigated by means of ELISA (von Willebrand Factor kit, Nodia BV, Amsterdam, The Netherlands).

Statistical Analysis

Variations over the study period were investigated using repeated measures ANOVA. To investigate differences between groups, continuous variables where compared

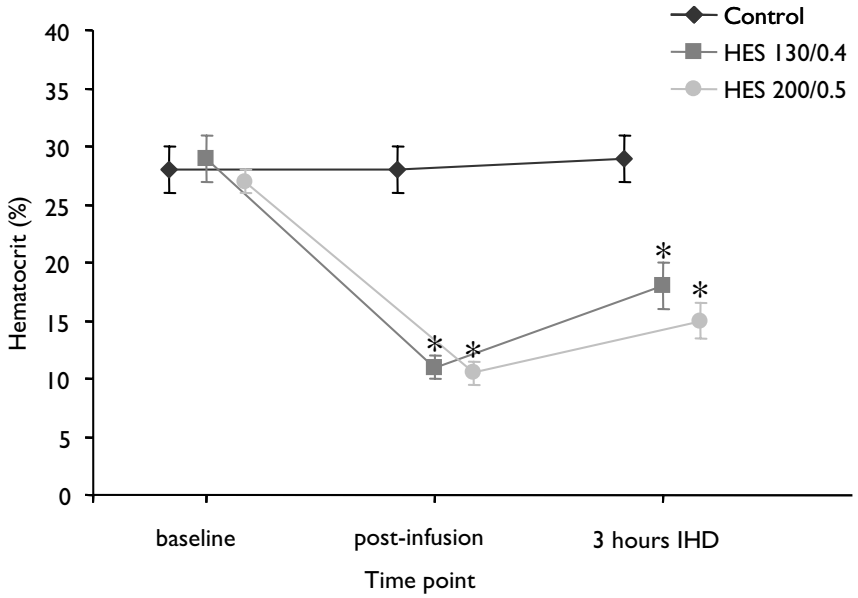

Figure 1. Hematocrit (%) variation during 3 h of acute isovolemic hemodilution, infused with either 3% HES 130/0.4 solution (n=6) or 3% HES 200/0.5 solution (n=6). The control group (n=3) is represented by sham-operated animals. The values are represented as mean (symbols) and standard error of the mean (bars). * $p<0.05$ vs. control.

by means of parametric (Student's T-Test) or nonparametric tests (Mann-Whitney). Correlation between nonparametric variables was performed with Spearman's correlation test. Results are presented as mean ± SEM (unless stated otherwise). For all analyses, a p-value ≤0.05 was considered to indicate statistical significance.

Results

Immediately post-infusion the hematocrit (Hct, Fig.1) reached 39.9 ± 1.9% of baseline values in HES 130/0.4 hemodiluted animals and 41.3 ± 2.2% of baseline values in HES 200/0.5 hemodiluted animals. Hct partially recovered by the end of the 3 experimental hours to 65.5 ± 5.9% and 57.9 ± 4.3% of baseline values, respectively. The extraordinary compensating capacity of the circulating number of erythrocytes was probably achieved by mobilization of spleen-trapped erythrocytes. This observation was supported by a smaller size and pale color of spleens in hemodiluted animals, as compared with those of the sham-operated animals. To exclude the possibility of hemoconcentration due to loss of infused fluid through urine or extra vascular extravasations, we performed plasma viscosity measurements. The baseline plasma

Table 1. Hemodynamic parameters

	Baseline	Post-infusion	3 hours IHD
MAP (mmHg)			
Control	71.8 ± 13.1	64.3 ± 6.3	53.3 ± 2.7[a]
HES 130/0.4	71.0 ± 16.6	46.1 ± 4.8[b]	48.3 ± 5.5[a]
HES 200/0.5	67.3 ± 9.0	52.6 ± 17.4	49.5 ± 5.5[a]
HR (beats/min)			
Control	100 ± 4	99 ± 1	106 ± 9
HES 130/0.4	90 ± 10	108 ± 13	138 ± 11[a,b]
HES 200/0.5	91 ± 9	106 ± 10	124 ± 22a
Arterial PO_2 (mmHg)			
Control	70.1 ± 2.8	70.7 ± 3.1	69.1 ± 1.6
HES 130/0.4	63.9 ± 6.5	70.1 ± 6.8	70.4 ± 5.3
HES 200/0.5	62.4 ± 7.1	68.4 ± 7.8	69.6 ± 7.1

[a]p<0.05 compared to baseline; [b]p<0.05 compared to control.

viscosity levels (1.7 ± 0.05 mPa·sec) dropped in the hemodiluted animals immediately after infusion (1.3 ± 0.06 mPa·sec HES 130/0.4; 1.4 ± 0.07 mPa·sec HES 200/0.5) and remained low until the end the experiment (1.3 ± 0.1 mPa·sec and 1.4 ± 0.06 mPa·sec, respectively), proving a comparable level of plasma dilution during the entire experiment.

Hemodynamics

Mean arterial pressure (MAP) and heart rate (HR) were monitored throughout the experiment (Table 1). MAP decreased gradually and significantly (p<0.001) in all animals during the experiment. Immediately post-infusion, MAP was significantly lower in the HES 130/0.4 group (p=0.024) than the control group; after 3 h of hemodilution no significant differences between groups were seen anymore. Heart rate increased gradually, with a stronger rise in hemodiluted animals (p=0.009). At the end of the experiment, the HES 130/0.4 group had significantly higher heart rates than the control group (p=0.024).

RBC aggregation

RBC aggregation (Fig. 2) decreased significantly after induction of hemodilution (p=0.002), with an overall lower aggregation index (AI) in the experimental animals as compared with sham-operated animals (p=0.001). In the HES 130/0.4 group, AI dropped to 39.2 ± 4.8% of baseline values and maintained low during the experiment with values of 37.05 ± 3.3% of baseline values at the end of experiment. In HES 200/0.5, AI declined post-infusion to 49.3 ± 5.9% of baseline values and maintained low with 47.7 ± 6.5% of baseline values at the end of experiment. Although the AI tended to be higher in the HES 200/0.5 group than in the HES 130/0.4 group, these differences were not statistically significant at any time point.

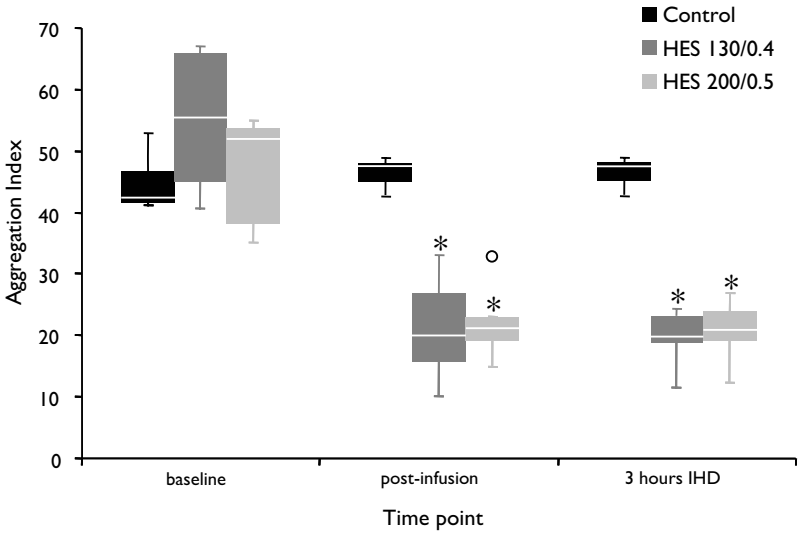

Figure 2. Red blood cell aggregation measured in fresh blood ex-vivo, as Aggregation Index in adult pigs during 3 h of acute isovolemic hemodilution (IHD), infused with either 3% HES 130/0.4 solution (n=6) or 3% HES 200/0.5 solution (n=6). The group (n=3) is represented by sham-operated animals. The boundary of the box closest to zero indicates the 25th percentile, the line within the box marks the median of 6 measurements, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. Symbol ° represents an outlier. * $p<0.05$ vs. control.

Hypoxic oxidative stress

Arterial pO_2 increased moderately but not significantly after hemodilution (Table 1), reflecting either an improvement in pulmonary gas exchange or decreased diffusional oxygen exit.

Plasma malondialdehyde (MDA, Fig. 3A) dropped significantly directly after infusion, due to the dilution effect. For sham operated animals (control group) the MDA values were 2.3 ± 0.05 µmol pre-anesthesia (baseline), and 2.7 ± 0.3 µmol after 3h anesthesia.

For HES 130/0.4 group the MDA values were 2.8 ± 0.08 µmol at baseline, 1.42 ± 0.09 µmol immediately after hemodilution, and 1.83 ± 0.1 µmol after 3h of hemodilution.

For the HES 200/0.5 group, MDA values were 2.8 ± 0.2 µmol at baseline, 1.19 ± 0.09 µmol immediately after hemodilution, and 1.58 ± 0.1 µmol after 3 h of hemodilution.

Relative increase in plasma MDA during the three experimental hours was comparable in sham-operated animals (0.34 ± 0.09 µmol), HES 130/0.4 infused animals (0.30 ± 0.16 µmol) and HES 200/0.5 infused animals (0.40 ± 0.18 µmol).

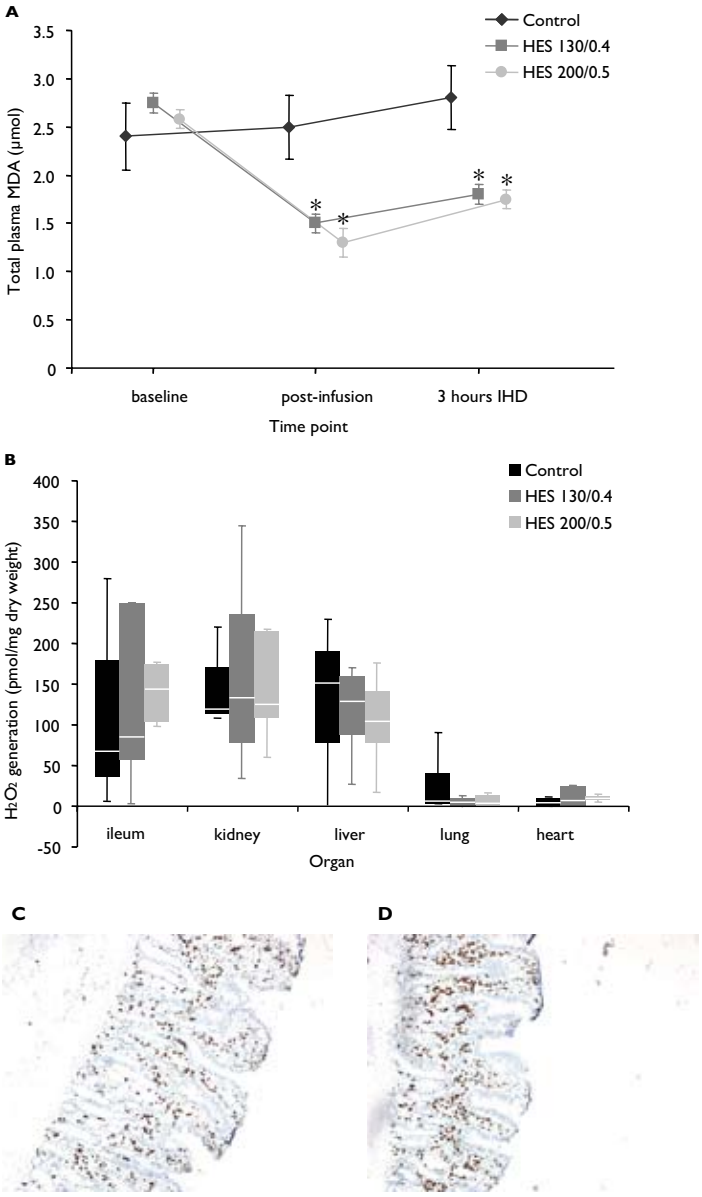

Figure 3. Hypoxic oxidative stress during 3 h of acute isovolemic hemodilution.
(A) Plasma malondialdehyde (MDA): The values are represented as mean (symbols) and standard error of the mean (bars); **(B)** Ex-vivo hydrogen peroxide (H_2O_2) production in heart, lung, kidney, liver and ileum tissue biopsies reflecting mitochondrial dysfunction in tissues pre-exposed to ischemia in vivo. Box plots graph data represent statistical values (see legend Fig 2). **(C)** Diaminobenzidine(DAB) staining of H_2O_2- producing cells (brown coloration) in paraformaldehyde-fixed frozen sections of ileum mucosa of hemodiluted and **(D)** sham-operated animals. * $p<0.05$ vs. control.

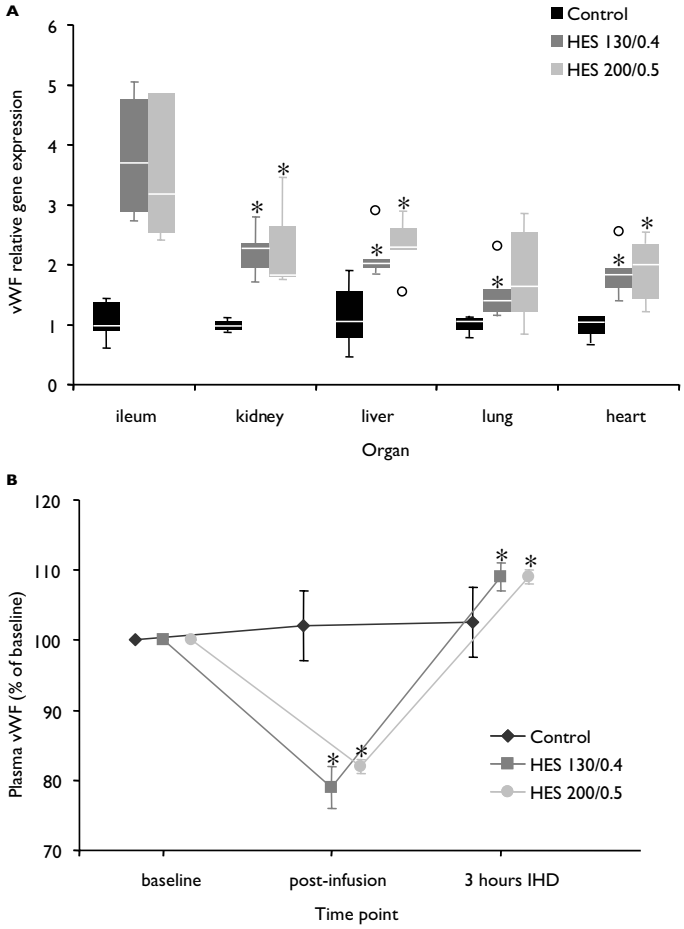

Figure 4. von Willebrand factor (vWF) **(A)** vWF relative gene expression of in the heart, lung, kidney, liver and ileum tissue biopsies at the end of the experiment. Box plots graph data represent statistical values (see legend Fig. 2). **(B)** vWF plasma concentrations. The values are represented as mean (symbols) and standard error of the mean (bars). * $p < 0.05$ vs. control.

All animals showed a statistically significantly higher H_2O_2 production in the abdominal organs (ileum, kidney and liver) than in heart and lung tissue (Fig. 3B). Oxygen radicals production was similar in all animals, with no statistically significant difference at any time point between hemodiluted and sham-operated animals.

DAB staining of H_2O_2 producing cells in the ileum was performed, as the ileum seemed to be one of the organs exposed to oxidative stress. Figure 3 shows a similar villi morphology, comparable staining intensity and distribution of H_2O_2 producing cells in both hemodiluted (Fig. 3C) and sham-operated animals (Fig. 3D).

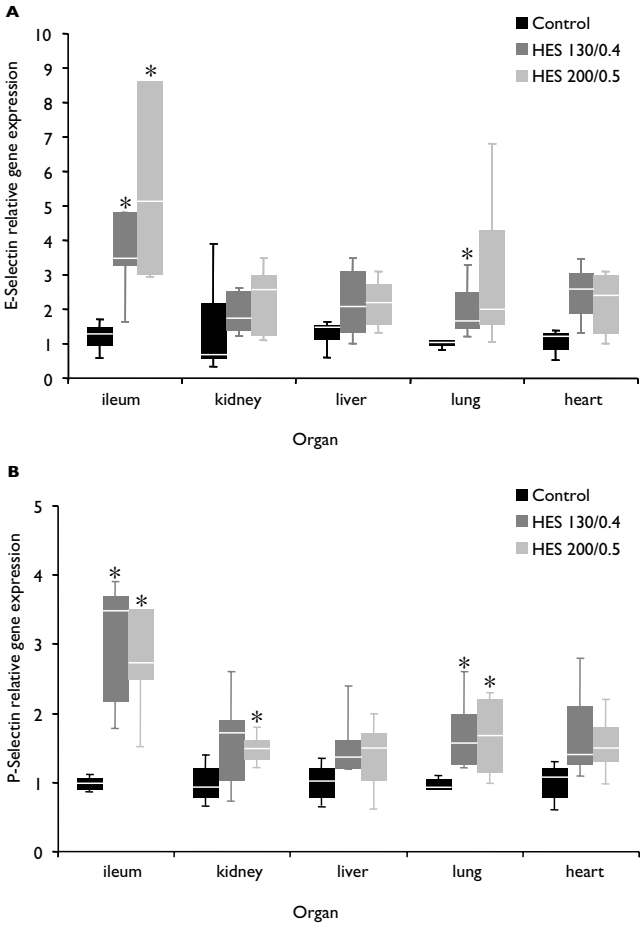

Figure 5. E-Selectin **(A)** and P-Selectin **(B)** relative gene expression of in the heart, lung, kidney, liver and ileum tissue biopsies at the end of the experiment. Box plots graph data represent statistical values (see legend Fig 2). * $p < 0.05$ vs. control.

Vascular endothelial activation

Von Willebrand Factor (vWF) mRNA after 3 h of hemodilution (Fig. 4A) was significantly up-regulated in HES 130/0.4 hemodiluted animals when compared with sham-operated animals in all organs studied (ileum, kidney, lung and heart p=0.024, liver p=0.048). A similar outcome was found in HES 200/0.5, with the exception of lung, where differences did not reach statistical significance (ileum, kidney, and heart p=0.024, liver p=0.048 and lung p=0.095). vWF mRNA responses did not differ between HES 130/0.4 and HES 200/0.5 treated animals.

Plasma vWF systemic release (Fig. 4B) reflects the information found at mRNA level. The relative increase in vWF plasma concentrations during three experimental

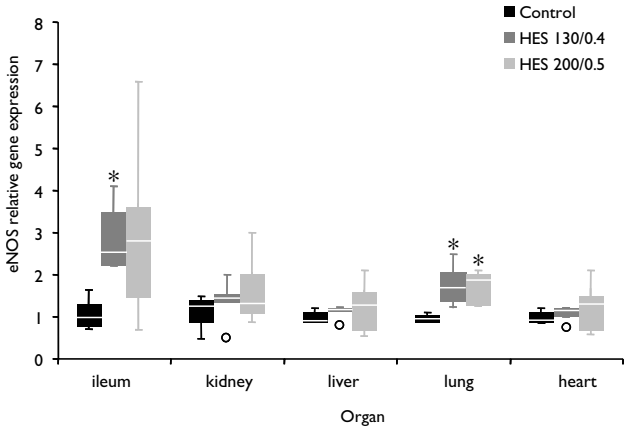

Figure 6. Endothelial nitric oxide synthase (eNOS) relative gene expression in the heart, lung, kidney, liver and ileum tissue biopsies at the end of the experiment. Box plots graph data represent statistical values (see legend Fig 2). * $p < 0.05$ vs. control.

hours in HES 130/0.4 group (30.32 ± 4.6%) and in HES 200/0.5 group (27.9 ± 1.3%) were significantly higher than the control values (0.1 ± 0.01%) in sham operated animals (p=0.024 for both comparisons).

E-Selectin mRNA after 3 h of hemodilution (Fig. 5A) was significantly up-regulated in the ileum and lung of HES 130/0.4 hemodiluted animals, as compared with the sham-operated ones (p=0.048, and p=0.024 respectively). HES 200/0.5 hemodilution resulted in significantly up-regulated E-Selectin mRNA in the ileum (p=0.024) when compared with control levels.

P-Selectin mRNA after 3 h of hemodilution (Fig. 5B) was significantly up-regulated in ileum and lung of both groups of hemodiluted animals (HES 130/0.4: ileum, lung p=0.024, HES 200/0.5: ileum p=0.024, lung p=0.048). Additionally for HES 200/0.5, levels measured in the kidney reached statistical significance when compared with control animals (p=0.048).

Endothelial nitric oxide synthase (eNOS) mRNA after 3 h of hemodilution (Fig. 6) was significantly up-regulated in ileum and lung in the of HES 130/0.4 group (p=0.024 for both organs). In the HES 20/0.5 group, eNOS was only significantly up-regulated in the lung (p=0.024).

Correlations

A statistically significant negative correlation was found between the RBC aggregation index post-infusion and different markers of endothelial activation measured after 3 h of hemodilution (Table 2).

Table 2. RBC aggregation and endothelial activation

Spearman's correlation		Aggregation index post-infusion
P Selectin- ileum	Correlation Coefficient	-0.614 (p=0.015)
vWF- ileum	Correlation Coefficient	-0.539 (p=0.038)
eNOS- ileum	Correlation Coefficient	-0.486 (p=0.066)
vWF- liver	Correlation Coefficient	-0.546 (p=0.035)
P Selectin- lung	Correlation Coefficient	-0.582 (p=0.023)
P Selectin- heart	Correlation Coefficient	-0.496 (p=0.060)
vWF- heart	Correlation Coefficient	-0.632 (p=0.011)

Discussion

Acute isovolemic hemodilution caused a dramatic drop in erythrocyte aggregation. This drop in RBC aggregation was followed by an immediate pro-thrombotic and pro-inflammatory endothelial activation in all vital organs. Erythrocyte aggregability correlated significantly with markers of endothelial activation suggesting a causality effect. RBC hyper-aggregation is nowadays a generally recognized pathogenic factor in disorders associated with macro and/or microvascular impairment, e.g. hypertension, diabetes mellitus, and chronic venous insufficiency (18-20). Hypo-aggregation of RBCs has never been described in a pathologic context. Given the impact of RBC aggregation on blood rheology, and thus on shear stress-dependent endothelial activation, low RBC aggregation might be an important pathogenic co-factor in endothelial activation during acute isovolemic hemodilution.

Hemodilution is expected to decrease the oxygen-carrying capacity of blood and oxygen delivery to the tissue. However, during moderate levels of hemodilution, reduction of the systemic hematocrit up to 50% is compensated with an increased blood flow and decreased diffusional oxygen exit from arterioles, resulting in augmented or maintained oxygen delivery to tissue (21). In an experimental animal study, Deem and colleagues showed that acute isovolemic hemodilution in healthy rabbits resulted in improved gas-exchange efficiency, as shown by higher arterial pO_2, lower alveolar-arterial pO_2 difference, and increased expired NO (22). They postulated that the improvement in oxygenation appeared to be related to increased uniformity of pulmonary blood flow, and/or an increase in concentration of the vaso- and bronchodilator substance NO. The present data support this assumption and consistently show an up-regulation of eNOS in the lung tissue during acute hemodilution.

In the present study changes in tissue oxygenation were quantified by assessing mitochondrial (dys)function in vital organs (heart, lung, kidney, liver and ileum) of hemodiluted animals. The production of superoxide and hydroxyl radicals, measured as H_2O_2 generation, was similar at all time points in sham-operated and hemodiluted

animals. This finding indicates that a similar hypoxic oxidative stress was present, and that oxygen delivery to the tissue was maintained during hemodilution. However, different organs seemed to have different exposure to hypoxia, with a more profound mitochondrial dysfunction in abdominal organs (ileum, kidney and liver) versus a preserved function of mitochondria in the myocardium and lung tissue. The results found in tissue biopsies were mirrored by the plasma MDA measurements, that showed similar relative increase in systemic lipid peroxidation products during three experimental hours when hemodiluted animals and sham-operated animals were compared. Given the negligible amount of hypoxic stress and the strong statistical correlation between red blood cell aggregation and markers of endothelial activation, low erythrocyte aggregation might be the trigger for endothelial activation.

In this experiment, acute isovolemic hemodilution resulted in immediate pro-thrombotic endothelial activation as shown by a systemic increase in plasma vWF levels. Analysis of vWF mRNA expression levels in different vital organs showed an up-regulation in heart, lung, kidney, liver and small intestine. In addition, low red blood cell aggregation was significantly associated with high vWF mRNA expression in heart, liver and ileum suggesting a causality effect.

RBC hypo-aggregation was associated with an up-regulation of endothelial adhesion molecules, E- and P-Selectins, especially in lung and small intestine. Selectins play important roles in the inflammatory responses by facilitating leukocyte rolling and leukocytes activation (23;24).

Although diluted blood was able to provide sufficient oxygenation and metabolic support, the data presented in this study show that acute isovolemic hemodilution triggers endothelial activation. An inflammation and coagulation prone endothelium will most certainly result in extra damage upon reperfusion of the graft. All this qualifies diluted donor blood as a suboptimal preservation solution. Optimization of normothermic preservation solutions is therefore necessary to be able to fully utilize the NMP potential. One strategy to improve the use of diluted donor blood in normothermic preservation might be to filter out certain cell types. Harper and colleagues showed that depleting leukocytes during whole blood normothermic kidney preservation resulted in improved kidney graft function after transplantation (25). Another approach might be to develop a novel, synthetic normothermic preservation solution. Such a solution might contain artificial oxygen carriers like perfluorocarbon chemicals for adequate oxygen delivery and several saccharides, aminoacids and vitamins for nutritional support. Additional colloids will prevent edema formation. The group of Kootstra has performed some promising experiments with an acellular synthetic normothermic preservation solution. By using their protocol called Exsanguinous Metabolic Support (EMS) they could successfully transplant canine kidneys that had suffered from 120 minutes of warm ischemia (26). Similar to the situation in the late sixties when cryoprecipitated donor plasma was replaced by the first static cold storage solution of G.M. Collins, history may repeat itself and a synthetic alternative for diluted donor blood will become available in the next decades.

Acknowledgments
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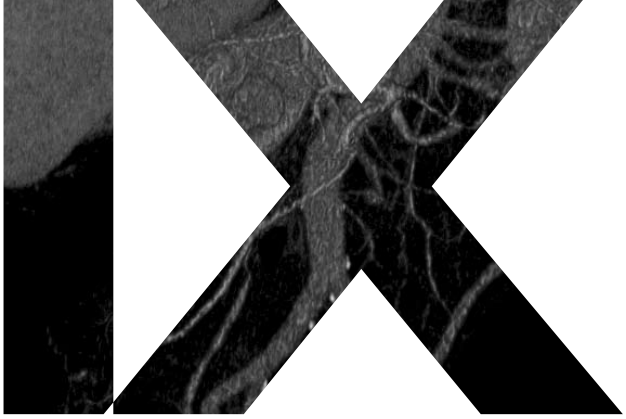


Chapter IX

Summary and future perspective

Summary

Clinical kidney and liver transplantation has evolved from an experimental procedure in the early 1950s to a routine clinical practice for treatment of end stage organ disease. With an increasing number of patients on the waiting list, future advances in this field are constrained by both the numbers of donor organs available as well as their quality. Improved road safety, the use of airbags and improvements in intensive care medicine have led to a plateau in the number of organ donors. In order to make ends meet, organ donation criteria have been expanded. As a result, the average deceased donor is nowadays older, has suffered from a cerebrovascular accident (CVA) rather than isolated head trauma, and therefore has more associated morbidity, which negatively influences graft quality. In order to close the widening gap between supply and demand the usage of organs derived from donation after cardiac death (DCD) is increasing in most countries. The combined effect of these negative changes in organ donor demographics has led to a decrease in organ viability and an increase in delayed graft function (DGF) after transplantation. A slow initiation of graft function after transplantation has a negative impact on a kidney recipient but is often fatal to a liver transplant recipient. While patients receiving a DGF kidney can be supported with dialysis, delayed function of a liver graft can only be treated with urgent re-transplantation since no adequate long-term artificial liver support is available yet. Limiting the incidence of DGF will have a major impact on transplantation medicine. Improving organ preservation, which is the topic of this thesis, might be a way to achieve this goal. Organ preservation allows bridging from donor to recipient and is therefore pivotal to transplantation logistics. Present day hypothermic organ preservation, however, is both friend and foe to organ viability. Hypothermia, which is currently the main stay of clinical organ preservation, protects the graft from ischemia/reperfusion injury by slowing down the metabolic demand. Unwanted harmful side-effects of hypothermia, however, include cell swelling, acidosis and the formation of reactive oxygen species which all injure the graft. Damage inflicted by the cold has to be counteracted by using tailor made preservation solutions. At this moment clinical organ preservation is most frequently performed with the static cold storage (CS) method using the University of Wisconsin cold storage solution (UW-CSS), which was developed in the late 1980s. Although static cold storage using UW-CSS allowed expansion of organ transplantation programs all over the world, it might well be that both CS and UW-CSS have reached their limits given the present composition of the donor pool. Improving organ preservation by using better preservation solutions or an improved preservation method will therefore help to reach the goal of reducing the incidence of DGF and thereby improving outcome after transplantation.

Chapter I

In the introduction of this thesis (*Chapter I*) the importance of maintaining organ viability after donation until transplantation is illustrated. To date, static cold storage using UW-CSS is the most widely used form of organ preservation. Although simple and effective, it is questionable whether this method is able to prevent deterioration

of organ quality in the present era with increasing numbers of organs retrieved from older, marginal and DCD donors. Basic principles involved in effective preservation and effective components and methods of organ preservation in abdominal organ transplantation are reviewed. A new preservation solution is Institut Georges Lopez-1 (IGL-1), which was developed at the Edouard Herriot Hospital in Lyon, France, in the late 1990s. The design of IGL-1 is based on UW-CSS with two important changes. First, in UW-CSS hydroxyethyl-starch (HES) is used as a colloid. Several groups, including our own, have shown that HES causes red blood cell aggregation during wash-out of the organ. This aggregational behaviour of HES negatively influences wash-out efficacy of UW-CSS. IGL-1 uses polyethylene glycol (PEG) as a colloidal substance, which does not induce red blood cell aggregation. The second important difference in the IGL-1 solution is the so called extracellular sodium/potassium ratio. Compared to UW-CSS, which has an intracellular sodium potassium/ratio, IGL-1 has a low potassium content. The high potassium content in UW-CSS is known to provoke an arterial vasospasm. IGL-1 might be able to reduce this effect. Apart from different solutions this chapter also focuses on a different preservation method: hypothermic machine perfusion (HMP). Although not a new approach -clinical organ preservation in the early 1960s started with HMP- this method might be able to improve preservation of the present day damaged donor organs. Despite the fact that HMP might be superior to static cold storage, organs are still exposed to hypothermia induced damage. Therefore, recent experimental studies point towards the benefits of normothermic machine perfusion (NMP) as a new perspective in organ preservation and transplantation.

Chapter II

In this chapter the rationale of the studies which are included in this thesis is presented. The first part of this thesis concerns static cold storage preservation experiments using IGL-1 in rat models of kidney preservation and transplantation, focussing on vasomotor function *(Chapter III)* and organ viability *(Chapter IV)*, and comparing this to UW-CSS. The second part focuses on the development and functional evaluation of the Groningen Machine Perfusion (GMP) system *(Chapter V)* and its subsequent application in a porcine kidney transplantation model *(Chapter VI)*. In November 2005 a randomized clinical trial was initiated within Eurotransplant to determine the efficacy of the HMP concept in clinical kidney preservation and transplantation. The first results of this clinical trial are presented in *Chapter VII*. In preparation of using the GMP system in a normothermic mode, the use of diluted donor blood as preservation solution is studied in a porcine model *(Chapter VIII)* with an emphasis on endothelial damage.

Chapter III

Although ischemia/reperfusion injury to kidney parenchyma has been extensively studied, little is known about the functional effects of warm and cold ischemia on the renal vascular bed. In this chapter, rat kidney preservation using IGL-1 was compared to UW-CSS with a special focus on vasomotor functions. The influence of warm

and cold ischemia on vasomotor functions was studied under controlled conditions in an isolated perfused kidney (IPK) model. Six groups of rat DCD donor kidneys were studied with increasing duration of warm ischemia of 0, 15 or 30 minutes followed by 0 or 24 h CS preservation in IGL-1 or UW-CSS at 4°C. Endothelial dependent vasodilation was studied using acetylcholine (ACH), smooth muscle cell constriction was assessed using phenylephrine (PE) and finally endothelial independent relaxation was tested using papaverine-sulphate. The experiments demonstrated that smooth muscle cells were significantly affected by cold ischemia showing a 50% reduction of PE mediated constriction after preservation. This rapid decline in smooth muscle cell function might be the first step towards intima hyperplasia as seen in late transplant dysfunction. Additional warm ischemia did not influence SMCs. After IGL-1 preservation, endothelial dependent vasodilation was negatively affected by both warm and cold ischemia. In the UW-CSS groups only the combination of warm and cold ischemia significantly reduced the ACH response. Both IGL-1 and UW-CSS rendered equal results after 24 h preservation. These results demonstrated that vasomotor functions are negatively influenced by the combination of warm and cold ischemia, as is clinically seen in DCD organs.

Chapter IV

In this chapter the effect of IGL-1 preservation on organ viability after preservation and transplantation in rats was studied and compared to the results obtained with UW-CSS. The influence of warm and cold ischemia on renal damage and graft function was evaluated in a rat Lewis-Lewis transplant model with a follow up of 14 days. Eight groups of donor kidneys were studied with warm ischemia of 0 and 15 minutes followed by 0 or 24 h cold storage preservation in IGL-1 or UW-CSS at 4°C. Blood was collected daily during the first week and at day 14. Recipients were placed in metabolic cages at day 4 and 14 after transplantation allowing urine collection and adequate measurement of glomerular filtration rate. Focussing on inflammation, reactive oxygen species production, proximal tubule damage, proteinuria, histology, and renal function after transplantation we could not show any relevant difference between IGL-1 and UW-CSS. Furthermore, the combination of 15 min warm ischemia and 24 h cold ischemia did not result in life sustaining kidney function after transplantation, irrespective of the used solution. These results confirm the findings in *Chapter III* and demonstrated that static cold storage preservation of ischemically damaged rat kidneys in either IGL-1 or UW-CSS render equal results after preservation and transplantation.

Chapter V

Since CS might have reached its limits in liver preservation, a new portable HMP system was developed in a close collaboration between our laboratory and the department of Biomedical Engineering at the University Medical Center Groningen. In this chapter, a prototype of the Groningen Machine Perfusion (GMP) system was evaluated with a focus on functionality and tissue morphology after 24 h preservation. Evaluation criteria included adequate pressure controlled perfusion, sufficient

oxygenation, a temperature of 0-4°C and sterile conditions. In a porcine heart beating donor model livers were preserved with the GMP system using University of Wisconsin machine perfusion solution (UW-MPS) or with CS using UW-CSS during 24 h. During HMP, livers were perfused with pump pressures set at 4 mmHg for the portal vein and 30/20 mmHg, at 60 BPM, for the hepatic artery. In the HMP group, pressure, flow and temperature were continuously monitored for 24 h. At time-points 0, 2, 4, 8, 12 and 24 h samples of UW-MPS solution were taken for measurement of partial oxygen pressure (pO_2) and lactate-dehydrogenase (LDH). Liver biopsies were taken for histology and electron microscopy; samples of ice, preservation solution, liver surface and bile were taken and cultured to determine sterility. During 24 h preservation using the GMP system temperature was maintained at 0-4°C. Arterial pO_2 was kept at 100 kPa throughout the experiment while venous pO_2 reached appropriate levels (>20 kPa) within 2 h. Histological evaluation showed a complete perfusion of the liver with no differences in tissue morphology when compared to CS livers. These results showed that the GMP system complied with the design criteria. The proof of principle will have to be demonstrated in a transplantation experiment.

Chapter VI

The merits of a new preservation method like the GMP system can only be properly assessed in a transplantation model that includes reperfusion of the graft. The porcine liver transplantation model is, however, a very demanding, complex and expensive experimental model. Therefore in this chapter the principle of HMP preservation using the GMP system is assessed in a porcine kidney transplantation model. A re-design of the liver GMP system was developed for kidney preservation and the aim of the present study was to examine the efficacy of this system compared to CS using UW-CSS. In a porcine autotransplantation model kidneys were retrieved and either cold stored in UW-CSS for 20 h at 4°C or subjected to HMP using UW-MPS at 4°C with two different pressure settings: 30/20 mmHg or 60/40mmHg. HMP at 30/20 mmHg was found to better preserve the viability of kidneys reflected by improved cortical microcirculation, less damage to the proximal tubule, less damage mediated by reactive oxygen species, less pro-inflammatory cytokine expression and better functional recovery after transplantation. However, high perfusion pressures (60/40 mmHg) resulted in higher expression of von Willebrand factor and monocyte chemotactic peptide-1 in post preservation biopsies and subsequent graft thrombosis in 2 kidneys. It is concluded that the GMP system improves kidney graft viability, however, perfusion pressures are critically important for outcome. In this experiment a perfusion pressure of 30/20 mmHg during HMP rendered the best results.

Chapter VII

Retrospective analyses have indicated that preservation by hypothermic machine perfusion (HMP) may lead to improved outcome after renal transplantation. However, there is a lack of sufficiently powered prospective studies to test the presumed superiority of HMP. In this chapter the results of an international prospective randomized controlled trial are reported. This trial is registered as an International Standard Randomized

Controlled Trial, number ISRCTN83876362. Kidney pairs of 336 consecutive deceased donors were enrolled. One organ was randomly assigned to HMP and the contralateral kidney to CS preservation. All 672 recipients were followed up for one year. The primary endpoint was delayed graft function (DGF). Secondary endpoints were functional DGF, duration of DGF, primary non-function, serum creatinine and creatinine clearance, acute rejection, calcineurin inhibitor toxicity, length of hospital stay, and graft and patient survival post-transplant.

No serious adverse events directly attributable to HMP were encountered. HMP significantly reduced the risk of DGF (HMP-arm 70/336, CS-arm 89/336; adjusted OR 0.57; p=0.01), and of functional DGF. In the event that DGF occurred, its duration was shorter after HMP. Serum creatinine values were lower for HMP kidney recipients in the first two weeks post-transplant. HMP reduced the risk of graft failure in the first year after transplantation (HR 0.37; p=0.02). One year graft survival was superior in the HMP group (98% versus 94%; p=0.03). No significant differences were found for the other secondary endpoints. It was concluded that in all common types of deceased donor kidney transplantation, HMP reduced the risk of DGF. In addition, HMP improved graft survival in the first year after transplantation.

Chapter VIII

While machine perfusion was shown to improve clinical kidney preservation, it is still based on hypothermia with its well known deleterious side effects. A new concept in organ preservation which does not induce hypothermia related injury is normothermic machine perfusion (NMP). NMP provides oxygen delivery at physiologic or near-physiologic temperature (37°C) and allows near-normal metabolism. Since traditional hypothermic preservation solutions cannot provide physiologic metabolic demands, diluted donor blood is often used as a preservation solution in experimental and clinical normothermic machine perfusion (NMP). In this chapter the effects of diluted blood were studied in a porcine isovolemic hemodilution (IHD) model. Acute IHD (30ml/kg exchange-transfusion with colloid-solutions) was induced in 15 pigs. Hypoxic oxidative stress (plasma malondialdehyde, ex-vivo oxygen radicals production in heart, lung, kidney, liver and ileum tissue biopsies), erythrocyte aggregation (LORCA), and endothelial activation (Real-Time quantitative RT-PCR on von Willebrand Factor (vWF), E- and P-Selectins, endothelial nitric oxide synthase gene-expression in tissue biopsies) were investigated. The production of superoxide and hydroxyl radicals, measured as H_2O_2 generation, was similar at all times in sham-operated and hemodiluted animals, proving a maintained oxygen delivery. Acute IHD was followed by a dramatic drop in erythrocyte aggregation and immediate pro-thrombotic (significant vWF mRNA up-regulation in heart, lung, kidney, liver and ileum) and pro-inflammatory (significant E-and P-Selectins mRNA up-regulation in lung and ileum) endothelial activation. Low erythrocyte aggregation was significantly correlated with increased mRNA-expression of vWF (heart, liver, ileum) and P-Selectin (lung, ileum and heart). The results suggest that low erythrocyte aggregation during hemodilution triggers endothelium-dependent thrombogenic and pro-inflammatory responses. Given the potential impact of inflammation and coagulation upon reperfusion of a normothermic preserved graft

the use of diluted donor blood as a normothermic preservation solution should be considered suboptimal.

Future perspective

Multidisciplinary research at the interface of organ preservation and biomedical engineering is both exciting and rewarding to the scientist. Equally important to the scientific significance is the fact that this type of applied science is directed at improving the quality of donor organs and thus the quality of life of transplant patients. The interaction of biology, technology and medicine creates a broad and challenging research area where bridges between the individual disciplines have to be built every day. Given the fact that the strength of a bridge is inversely correlated to its length we can consider ourselves fortunate in Groningen. The small distance and close collaboration between the departments of BioMedical Engineering, the Surgical Research Laboratory and clinical units have created a strong and stable research environment with a promising future.

Improving static cold storage preservation

This thesis describes studies which aim to enhance organ preservation in order to improve outcome after kidney and liver transplantation. A key factor in organ preservation is the mismatch between metabolic demand and supply which occurs when an organ is disconnected from systemic circulation. Current organ preservation strategies aim at limiting the metabolic demand of the organ by cooling to 0-4°C. This causes a decrease in metabolism of approximately 90%. The collateral damage of hypothermia, however, is limiting further improvement of hypothermia-based organ preservation techniques. Novel strategies should focus on a reduction of metabolic demand or improved metabolic support or combine both approaches.

Instead of lowering metabolism by hypothermia, new pathways will have to be elucidated which will lead to a reversible induction of 'hypo-metabolism'. Much can be learned from nature at this point. Novel insights to improve organ preservation may arise from studies concerning hibernating animals that experience low body temperature and metabolic depression for longer periods of time. Hibernation is a condition involving dramatic changes in the function of organ systems and individual tissues. An important aspect of this process is a significant reduction in both energy production and energy consumption. During hibernation the body temperature decreases close to ambient temperature (0-10°C), body functions are reduced to a minimum, metabolism is depressed and animals enter a lethargic state lasting several days or weeks. In this state, organisms are resistant to environmental stresses including temperature extremes and oxygen deprivation. Interestingly, damage to livers of non-hibernating (summer) ground squirrels after 72 h of cold storage is much greater than for hibernating ground squirrels. Thus, within the same species, there is a switch from a less tolerant (summer) phenotype to a super tolerant (hibernating) phenotype. This suggests that systemic and cellular pathways that increase organ tolerance to cold

ischemia/reperfusion are repeatedly induced within the same individual on an annual basis. Elucidation of the increased tolerance to cold storage displayed by torpid hibernators could have a profound effect on organ preservation. A novel compound that is able to induce a hibernation-like state in non-hibernating species *(Szabó C, Nature Reviews 2007)* is hydrogen sulphide (H_2S). This colourless and water soluble gas with a characteristic smell of rotten eggs has primarily gained attention as being a toxic gas emanating from swamps. H_2S is, however, also produced in mammals including humans and belongs to a family of biological mediators which are called gasotransmitters. Well known other gasotransmitters are carbon monoxide (CO) and nitric oxide (NO). H_2S has a wide variety of biological effects resulting in cytotoxic effects or cytoprotective actions depending on dose and target. Its action as an inhibitor of cytochrome c oxidase, a critical step in ATP synthesis, is probably responsible for the H_2S induced hibernation-like state. When mice were exposed to low concentrations of H_2S gas, a dose-dependent reduction in the core body temperature and metabolic rate of the animals were observed. Their breathing rate slowed from 120 breaths per minute to fewer than 10. Body temperature reached as low was 15°C. When H_2S exposure ended, animals fully recovered without notable permanent damage. Given these effects of H_2S, the application of this gas during (machine) preservation needs to be tested. In the GMP system H_2S can be given through the oxygenator or by coating the tubing or organ chamber with an H_2S-donor. When applying H_2S in static applications, coating of the organ bag might be an attractive way to deliver this gas during organ preservation. It is likely that, similar to the situation in squirrels, organs in a hibernation-like state will be more tolerant to ischemia/reperfusion injury. Many questions regarding the small therapeutic window of H_2S and the late effects of H_2S exposure have to be answered before clinical organ preservation can benefit from metabolic management by H_2S. Also, by that time, some anti-odour will be available to protect the transplant team from the smell of rotten eggs.

Future developments concerning the Groningen Machine Perfusion system

In this thesis the GMP prototypes for liver and kidney preservation were designed and tested in a liver preservation and a kidney autotransplantation model. While in kidney preservation the GMP system proved superior over CS, this data is lacking for liver transplantation. In *Chapter VI*, where we tested the GMP system for kidney preservation and autotransplantation, pristine kidneys were used that had neither suffered from warm ischemia nor from allo-immune responses after transplantation. Future experiments should include DCD kidney and liver transplantation in an allo-transplantation model. Furthermore, a direct comparison between the GMP system and other commercially available kidney HMP devices should be made. This will allow assessment of the contribution of perfusate oxygenation and centrifugal pulsatile perfusion which are unique for the GMP system.

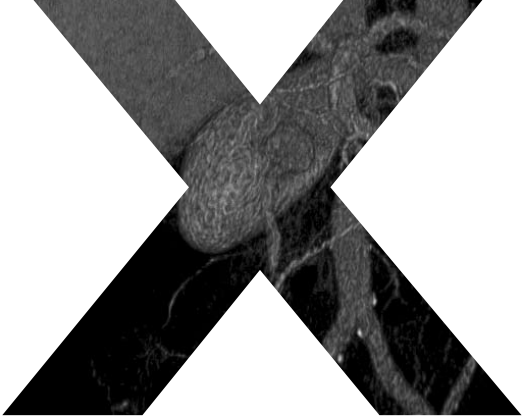
Many authors have stated that renal vascular resistance (RVR) changes during HMP might be useful for viability prediction. Although some retrospective data is available the data and samples from the randomized controlled clinical trial *(Chapter VII)* will allow verification of this hypothesis and identification of new viability markers.

Figure 1. Prototype of the Extra Coporeal Organ Procurement System (ECOPS).

Based on the recorded RVR during HMP and clinical outcome after transplantation, correlations can be calculated. This will answer the question whether RVR is truly a predictor for outcome and which cut-off levels for RVR changes should be applied. Trial samples from the UW-MPS at different time points can be used for the detection of novel biomarkers, allowing a more precise prediction of organ viability after transplantation.

Instead of lowering the metabolic demand future strategies could also aim at more metabolic support during organ preservation. If one could offer physiologic levels of oxygen, nutrients and removal of metabolites the insult to the organ will be as low as possible. Some initial results of experimental studies using the GMP system for normothermic preservation (NMP) of porcine livers at the University of Barcelona, Spain, are promising. Diluted donor blood was used as preservation solution in these experiments. Compared to CS, significant less parenchymal damage and a better synthetic function after liver transplantation were seen. Application in the clinical setting is currently limited because of the voluminous set-up and technical complexity. Future studies should address these problems in close conjunction with the used preservation solution. As shown in *Chapter VIII* of this thesis, diluted blood causes endothelial activation and may therefore disqualify as a preservation solution. Novel solutions for NMP may be synthetic and contain culture media compounds for metabolic support, colloids for edema prevention and oxygen carriers.

While studies in this thesis focussed on improving preservation of the isolated organ, optimal organ preservation should start earlier, i.e. in the donor. Optimizing organ procurement techniques will help to improve organ viability after preservation. In current clinical practice 6-14 liters of preservation solution at 0-4°C is rinsed through the vasculature of the donor at a pressure of 75-100 cm H_2O to wash-out blood and cool the organs. Application of a modified version of the GMP system for this purpose would allow a pressure controlled, oxygenated and continuous wash-out during donor surgery (Fig. 1). Instead of isolated perfusion of the kidney or liver, the entire abdominal compartiment of the donor can be perfused by cannulating the aorta and caval vein. By using this so called 'in-situ machine perfusion' the availability of oxygen would provide more metabolic support, while the continuous circulating preservation solution could result in improved wash-out and better cooling of all organs. The combined effect would be a reduction of the mismatch between metabolic demand and supply. When isolated organ preservation is then continued using HMP an optimal and technical feasible preservation strategy is available for every day clinical practice.



Chapter X

Samenvatting en toekomstperspectief

Samenvatting

Orgaantransplantatie heeft in de afgelopen decennia grote vooruitgang geboekt bij de behandeling van eindstadium nier- en leverfalen. Nier- en levertransplantaties worden tegenwoordig wereldwijd routinematig uitgevoerd en bieden goede overlevingskansen. Helaas dreigt de transplantatiegeneeskunde slachtoffer van haar eigen succes te worden, met als gevolg dat de vraag naar donororganen het aantal beschikbare organen ruim overstijgt waardoor de wachtlijsten gestaag groeien. Het donorpotentieel is door een toegenomen verkeersveiligheid en verbeteringen op de intensive-care afdelingen al jaren stabiel. Om de discrepantie tussen vraag en aanbod zo klein mogelijk te houden zijn orgaandonatiecriteria de afgelopen jaren verruimd. Wanneer we de huidige 'gemiddelde' donor vergelijken met die van een aantal jaren geleden valt een aantal zaken op. Ten eerste zijn donoren tegenwoordig ouder. Ten tweede is de doodsoorzaak tegenwoordig vaker een cerebrovasculair accident (CVA) dan een geïsoleerd hoofdtrauma. En ten slotte is er tegenwoordig sprake van meer co-morbiditeit. Hoewel het merendeel van de donororganen nog steeds afkomstig is van hersendode donoren, neemt het gebruik van organen die gedoneerd zijn na hartstilstand (donation after cardiac death; DCD) toe. Deze DCD organen zijn echter van mindere kwaliteit dan organen afkomstig van een hersendode donor.

Een lagere orgaankwaliteit leidt tot een verhoogde incidentie van het vertraagd op gang komen van het orgaan in de ontvanger (delayed graft function; DGF). In het geval van niertransplantaties resulteert DGF als gevolg van de benodigde intensieve zorg in een tijdelijk lagere kwaliteit van leven voor de ontvanger. Tevens leidt DGF tot hogere kosten voor de samenleving en een kortere overleving van het transplantaat. Door middel van nierdialyse kan de ontvanger van een donornier tijdens DGF in leven worden gehouden. Leverdialyse is echter onvoldoende ontwikkeld, zodat een ernstige DGF bij de levertransplantatiepatiënt alleen met een acute re-transplantatie behandeld kan worden.

Het beperken van de incidentie van DGF na transplantatie is dan ook cruciaal voor een verdere verbetering van de transplantiegeneeskunde. Verbeteren van orgaanpreservatie, het onderwerp van dit proefschrift, is een manier om dit te bereiken. Orgaanpreservatie heeft als doel de kwaliteit van het orgaan na uitname en tijdens transport van donor- naar ontvangerziekenhuis te behouden. De op dit moment gebruikte statische koude preservatietechniek (cold storage; CS) is gebaseerd op het afremmen van het celmetabolisme door het orgaan onder hypotherme condities (4°C) te bewaren. Hypothermie heeft echter ook een aantal nadelige effecten: het veroorzaakt cel-oedeem, acidose (verzuring) en initieert de productie van zuurstofradicalen. Om deze effecten zoveel mogelijk tegen te gaan zijn preservatievloeistoffen ontwikkeld die een aantal specifieke celbeschermende componenten bevatten. Zo werd na jaren van systematisch onderzoek in 1986 door Belzer en collega's in de Verenigde Staten de 'University of Wisconsin cold storage solution' (UW-CSS) ontwikkeld. Op basis van een in 1989 uitgevoerde prospectief gerandomiseerde multicenter studie in Europa werd duidelijk dat preservatie met UW-CSS resulteerde in een betere korte en lange termijn functie van donornieren in vergelijking met de EuroCollins oplossing,

de toenmalige gouden standaard. Aansluitend werd bekend dat UW-CSS ook voor lever, dunnedarm en pancreas de meest geschikte preservatievloeistof was. Sindsdien wordt voor klinische orgaanpreservatie UW-CSS het meest gebruikt.

Het is de vraag of CS met UW-CSS nog voldoet aan de eisen van deze tijd. Immers, de huidige 'gemiddelde' donor is ouder en heeft meer co-morbiditeit dan de 'gemiddelde' donor in het tijdperk waarin UW-CSS werd ontwikkeld. De hypothese van dit proefschrift is dat het verbeteren van orgaanpreservatie door gebruik van nieuwe vloeistoffen of van nieuwe preservatietechnieken bijdraagt aan het verlagen van de incidentie van DGF.

Hoofdstuk I

In dit inleidende hoofdstuk wordt het belang van orgaanpreservatie in de transplantatieketen benadrukt. Op dit moment worden de meeste organen met behulp van de statische koude preservatietechniek in UW-CSS gekoeld bewaard. Alhoewel deze techniek simpel en effectief is, is het zeer de vraag of deze methode in het huidige donortijdperk - met een slechtere orgaankwaliteit - nog afdoende werkt. Mogelijkerwijs zijn de huidige donoren gebaat bij een andere preservatietechniek. In dit hoofdstuk worden enkele concepten en criteria voor adequate abdominale orgaanpreservatie besproken. Eind jaren negentig werd door het Edouard Herriot Hospital in Lyon, Frankrijk een nieuwe preservatievloeistof ontwikkeld, de zogenaamde Institut Georges Lopez-1 (IGL-1) vloeistof. IGL-1 is gebaseerd op het UW-CSS concept, maar kent een tweetal belangrijke wijzigingen. In UW-CSS wordt hydroxyethyl zetmeel (hydroxyethyl starch; HES) als colloïd gebruikt. Verschillende groepen, waaronder wijzelf, hebben aangetoond dat het gebruik van HES aggregatie van rode bloedcellen tijdens het uitspoelen van het donororgaan kan veroorzaken. In IGL-1 wordt polyethyleenglycol (PEG) gebruikt als colloïd. PEG leidt, in tegenstelling tot HES, niet tot aggregatie van rode bloedcellen. Het tweede verschil tussen de beide vloeistoffen is het kaliumgehalte. Het hoge kaliumgehalte in UW-CSS kan leiden tot een vasospasme (vaatvernauwing), hetgeen een nadelig effect op preservatie kan hebben. IGL-1 heeft een lager kaliumgehalte dan UW-CSS waardoor er tijdens uitspoelen en preservatie geen spasme optreedt.

Naast diverse vloeistoffen behandelt dit hoofdstuk ook een andere preservatiemethode: hypotherme machinale preservatie (HMP). HMP werd in de jaren zestig van de vorige eeuw als orgaanpreservatietechniek gebruikt, maar vervolgens in de zeventiger jaren grotendeels door CS vervangen. Echter, door de huidige donororgaanproblematiek is voor HMP een hernieuwde belangstelling ontstaan. Om te voorkomen dat donororganen nog steeds blootgesteld worden aan de beschadigende effecten van hypothermie, zijn diverse onderzoeksgroepen in een experimentele setting bezig met het ontwikkelen van normotherme (37°C) machinale preservatie als een nieuwe modaliteit in orgaanpreservatie.

Hoofdstuk II

In dit hoofdstuk worden de doelstellingen van de verschillende studies die deel uitmaken van dit proefschrift beschreven. Het eerste gedeelte van dit proefschrift

bestaat uit experimenten in het rattenmodel naar het effect van IGL-1 op de renale vaatfunctie *(Hoofdstuk III)* en nierfunctie *(Hoofdstuk IV)* in vergelijking tot UW-CSS. Het tweede gedeelte van dit proefschrift bestaat uit de ontwikkeling en functionele evaluatie van het Groningse Machinale Perfusie (GMP) systeem *(Hoofdstuk V)* en de toepassing hiervan in een niertransplantatiemodel bij het varken *(Hoofdstuk VI)*. In november 2005 werd begonnen met een gerandomiseerde klinische studie binnen de Eurotransplant regio om de effectiviteit van HMP in nierpreservatie en -transplantatie te onderzoeken. De eindresultaten van deze studie zijn weergegeven in *Hoofdstuk VII*. In voorbereiding op normotherme preservatie met het GMP systeem wordt in *Hoofdstuk VIII* het effect van verdund donorbloed als preservatievloeistof op het endotheel in een varkensmodel onderzocht.

Hoofdstuk III

Alhoewel de effecten van ischemie/reperfusieschade op het nierparenchym uitgebreid bestudeerd zijn, is er relatief weinig bekend over de functionele effecten van warme en koude ischemie op het renale vaatbed. In dit hoofdstuk worden de functionele vasomotor-effecten van IGL-1 preservatie van de nier in een rattenmodel vergeleken met UW-CSS preservatie. De invloed van warme en koude ischemie op vasomotor-functies werd onder gecontroleerde condities in kaart gebracht met behulp van het geïsoleerd geperfundeerde niermodel (isolated perfused kidney; IPK). Zes groepen DCD donornieren met oplopende warme ischemietijden van 0, 15 of 30 minuten, gevolgd door 0 of 24 uur CS in IGL-1 of UW-CSS bij 4°C werden bestudeerd. Toegediende oplopende doseringen acetylcholine resulteerden in endotheel afhankelijke vasodilatatie. Phenylephrine injecties gaven gladde spiercel constrictie te zien en tot slot werd endotheel onafhankelijke relaxatie door papaverine geïnitieerd. De experimenten lieten zien dat gladde spiercellen significant minder functioneerden na blootstelling aan 24 uur CS, onafhankelijk van de gebruikte vloeistof. De snelle functionele achteruitgang van gladde spiercellen na 24 uur CS zou mogelijk de eerste stap kunnen zijn in de ontwikkeling van intima hyperplasie zoals wordt gezien bij chronisch transplantaatfalen. Warme ischemie had geen nadelig effect op de functie van gladde spiercellen. Na preservatie in IGL-1 resulteerde geïsoleerde warme ischemie en de combinatie van warme en koude ischemie in een afgenomen respons op acetylcholine. In de UW-CSS groepen leidde alleen de combinatie van warme en koude ischemie tot een significante afname in acetylcholine respons. De resultaten laten zien dat de combinatie van warme en koude ischemie, zoals klinisch gezien wordt in een DCD donor, vasomotor-functies nadelig beïnvloeden. Er was na 24 uur preservatie geen verschil in vasomotor-functies tussen IGL-1 en UW-CSS gepreserveerde nieren.

Hoofdstuk IV

In dit hoofdstuk wordt de nierfunctie na transplantatie volgend op 24 uur CS preservatie in IGL-1 vergeleken met de resultaten na 24 uur UW-CSS preservatie in een DCD rattenmodel. De effecten van warme en koude ischemie werden onderzocht in een Lewis-Lewis transplantatiemodel met 14 dagen overleving. Acht groepen

donornieren met warme ischemie perioden van 0 of 15 minuten, gevolgd door 0 of 24 uur CS preservatie bij 4°C in IGL-1 of UW-CSS werden bestudeerd. Tijdens de eerste postoperatieve week werd dagelijks bloed afgenomen en daarna op dag 14 na transplantatie. Door de ontvangers op dag 4 en 14 na transplantatie in zogenaamde metabole kooien te plaatsen, kon urine gecontroleerd worden opgevangen. De glomerulaire filtratie kon hierdoor nauwkeurig worden gemeten. Er was geen verschil tussen IGL-1 en UW-CSS preservatie in ontsteking, zuurstofradicaal productie, proximale tubulus schade, eiwitlekkage, histologie en nierfunctie na transplantatie. Onafhankelijk van de gebruikte preservatievloeistof, resulteerde de combinatie van 15 minuten warme ischemie en 24 uur koude ischemie in zeer ernstige nierbeschadiging met de dood van de ontvanger tot gevolg. Net als in *Hoofdstuk III* werd er na preservatie en transplantatie van rattennieren geen verschil tussen IGL-1 en UW-CSS aangetoond.

Hoofdstuk V

In ons laboratorium werd in samenwerking met de afdeling BioMedical Engineering van het Universitair Medisch Centrum Groningen een nieuw draagbaar HMP systeem voor leverpreservatie ontwikkeld. In dit hoofdstuk wordt de evaluatie van het prototype van het Groningse Machinale Perfusie (GMP) systeem beschreven. De functionaliteit werd beoordeeld op: drukverloop, oxygenatie-graad, temperatuurverloop, steriliteit en histologie van de organen na 24 uur preservatie. In een varkensmodel zonder additionele warme ischemie, werden zes levers uitgenomen en gepreserveerd met behulp van het GMP systeem. Voor het GMP systeem werd de 'University of Wisconsin machine perfusion solution' (UW-MPS) gebruikt. In de controlegroep werden twee levers met behulp van CS in UW-CSS gedurende 24 uur gepreserveerd. Perfusie vond plaats met een constante druk van 4 mmHg in de vena porta en een pulserende druk van 30/20 mmHg en 60 slagen per minuut in de arteria hepatica. In de HMP groep vond continue druk-, flow- en temperatuurregistratie plaats. Na 0, 2, 4, 8, 12 en 24 uur werden monsters van de UW-MPS genomen om de partiële zuurstofspanning (pO_2) en lactaatdehydrogenase (LDH) concentratie te meten. Leverbiopten werden zowel centraal als perifeer genomen ten behoeve van histologisch onderzoek (licht- en elektronenmicroscopie). Afgenomen monsters van het ijs, de preservatievloeistof, het leveroppervlak en gal werden op kweek gezet om eventuele bacteriegroei aan te tonen. De resultaten lieten zien dat het GMP systeem gedurende 24 uur in staat was de temperatuur op 0-4°C te houden. De arteriële pO_2 was vanaf het begin boven de 100 kPa. De veneuze zuurstofspanning was na 2 uur perfusie op niveau (>20 kPa). Bestudering van de histologie liet een complete perfusie van de lever zien zonder aantoonbare morfologische verschillen tussen HMP en controle levers. Het GMP systeem voldeed aan de vooraf opgestelde toetsingscriteria. Echter, het bewijs van superioriteit van het geoxygeneerde HMP principe zal in een transplantatie-experiment moeten worden geleverd.

Hoofdstuk VI

Zoals hierboven reeds beschreven kan de meerwaarde van een nieuwe preservatiemethode zoals het GMP systeem alleen maar worden aangetoond in

een transplantatiemodel. Een levertransplantatie bij het varken is echter een zeer ingewikkelde, kostbare en complexe experimentele procedure. Daarom wordt in dit hoofdstuk het principe van het GMP systeem aangetoond in een niertransplantatiemodel bij het varken. Een herontwerp van het GMP systeem resulteerde in een prototype dat geschikt was voor machinale nierpreservatie. Het doel van deze studie was om het transplantatieresultaat na preservatie met het GMP systeem te vergelijken met CS. In een autotransplantatiemodel werden varkensnieren uitgenomen en verdeeld in drie groepen. Nieren werden gedurende 20 uur gepreserveerd bij 0-4°C. In de eerste groep vond preservatie plaats met CS in UW-CSS. In de tweede groep werden nieren gepreserveerd met het GMP systeem met een pulserende druk van 30/20 mmHg en 60 slagen per minuut. In de laatste groep werd een druk van 60/40 mmHg gebruikt. HMP met een druk van 30/20 mmHg resulteerde in een beter behoud van nierkwaliteit. Een betere microcirculatie van de cortex, minder schade aan de proximale tubulus, minder zuurstofradicaal productie, minder pro-inflammatoire gen-expressie en een beter functioneel herstel in vergelijking met CS preservatie konden worden aangetoond. HMP met een perfusiedruk van 60/40 mmHg resulteerde echter in endotheelschade. Het gevolg was een statistisch significant hogere expressie van von Willebrandfactor en monocyte-chemotactic-peptide-1 in de post-preservatie biopten van deze groep. Niertrombose trad vervolgens op in twee van deze nieren. Geconcludeerd werd dat het GMP systeem varkensnieren beter preserveerde dan CS. Perfusiedrukken tijdens HMP zijn echter van cruciaal belang. In dit experiment gaf een perfusiedruk van 30/20 mmHg de beste resultaten.

Hoofdstuk VII

Alhoewel diverse retrospectieve studies suggereren dat HMP beter is dan CS, zijn er tot op heden geen prospectieve klinische studies die deze twee methoden voor nierpreservatie vergelijken. In dit hoofdstuk worden de eindresultaten van een gerandomiseerde multicenter studie gerapporteerd. Deze studie is geregistreerd als een 'International Standard Randomized Controlled Trial', nummer ISRCTN83876362. Nierparen (672 nieren) van 336 opeenvolgende overleden donoren konden worden geïncludeerd in deze studie. Van dezelfde donor werd één van beide nieren gerandomiseerd voor HMP. Preservatie van de andere nier van dezelfde donor vond vervolgens door middel van CS plaats. De 672 ontvangers van deze nieren konden allemaal worden vervolgd. Het primaire eindpunt was het vertraagd op gang komen van de nier (DGF). Secundaire eindpunten waren functionele-DGF, duur van de DGF, primaire non-functie, serum creatinine en creatinine-klaring, acute rejectie, calcineurine remmer toxiciteit, duur van de ziekenhuisopname en transplantaat- en patiëntoverleving na transplantatie. HMP resulteerde in een significante reductie van het optreden van DGF (HMP: 70/336, CS: 89/336; OR 0.57; p=0.01). Indien er sprake was van DGF, duurde dit bij HMP gepreserveerde nieren korter dan bij CS. Serum creatinine waarden van HMP gepreserveerde nieren waren lager dan CS nieren in de eerste twee weken na transplantatie. Tevens verkleinde HMP het risico op transplantaatfalen in het eerste jaar na transplantatie (HR 0.37; p=0.02). De transplantaatoverleving in het eerste jaar was beter bij HMP gepreserveerde nieren

(98% versus 94%; p=0.03). Voor de overige secundaire eindpunten werden geen significante verschillen gevonden. Samenvattend kan gesteld worden dat HMP het risico op DGF in alle typen overleden donoren verkleind. Verder resulteert HMP in een betere transplantaatoverleving in het eerste jaar na transplantatie.

Hoofdstuk VIII

In het voorgaande hoofdstuk is beschreven dat hypotherme machinale preservatie leidt tot betere klinische nierpreservatie. De HMP methodiek is echter nog steeds gebaseerd op hypothermie. Dit betekent dat alle nadelige effecten van hypothermie nog steeds een rol spelen. Een nieuw concept in orgaanpreservatie is normotherme machinale preservatie (NMP). Gedurende NMP wordt de temperatuur van het orgaan op 37°C gehouden en wordt het metabolisme nauwelijks geremd. Tijdens NMP treedt vanzelfsprekend geen hypotherme schade op. Omdat de huidige (hypotherme) presevatievloeistoffen onvoldoende in staat zijn om het hogere metabolisme en zuurstofverbruik tijdens NMP te ondersteunen wordt verdund donorbloed vaak gebruikt als preservatievloeistof. In dit hoofdstuk worden de effecten van verdund bloed (haemodilutie) als preservatievloeistof beschreven. Gelet op de gevoeligheid van het endotheel op verschillen in rheologie, veronderstellen we dat een lage rode bloedcelaggregatie zal leiden tot activatie van het endotheel na isovolemische haemodilutie (IHD). Inductie van acute IHD (30 ml/kg wisseltransfusie met colloïdale vloeistoffen) vond plaats in 15 varkens. Hypoxische schade (plasma malondialdehyde, ex-vivo zuurstofradicaal productie in hart-, long-, nier-, lever- en ileumbiopten), rode bloedcel aggregatie (LORCA) en endotheel activatie (Real-Time quantitatieve RT-PCR voor von Willebrandfactor (vWF), E- en P-selectines, eNOS gen expressie in weefselbiopten) werden onderzocht. De vorming van superoxide en hydroxyl-radicalen, gemeten door H_2O_2-productie, was op alle tijdspunten en in alle groepen gelijk. Dus, ondanks de haemodilutie, was er sprake van adequate oxygenatie van de weefsels. Acute IHD resulteerde in een verlaging van rode bloedcelaggregatie en een onmiddellijke pro-trombotische (significante vWF mRNA opregulatie in hart, long, nier, lever en ileum) en pro-inflammatoire (significante E- en P-selectines mRNA opregulatie in long en ileum) endotheel activatie. Een lage rode bloedcel aggregatie was significant gecorreleerd met een toegenomen mRNA-expressie van vWF (hart, lever en ileum) en P-selectine (long, ileum en hart). Deze resultaten suggereren dat een lage rode bloedcel aggregatie een endotheel afhankelijke trombogene en pro-inflammatoire respons kan induceren. Een trombogeen en pro-inflammatoir endotheel resulteert tijdens de reperfusiefase van een getransplanteerd orgaan waarschijnlijk in additionele schade door een heftigere immuunrespons van de ontvanger. Al met al werd geconcludeerd dat het gebruik van verdund donorbloed als preservatievloeistof voor NMP als suboptimaal moet worden betiteld.

Toekomstperspectief

Het verrichten van onderzoek op het grensvlak van orgaanpreservatie en biomedische technologie biedt fantastische uitdagingen voor de onderzoeker. Door de interactie van biologie, technologie en geneeskunde ontstaat een breed onderzoeksgebied waarbij kruisbestuivingen van en vanuit de diverse disciplines dagelijks plaatsvinden. Ervan uitgaande dat de kans op bestuiving omgekeerd evenredig is met de afstand, kunnen we ons in Groningen gelukkig prijzen. De geringe afstand tussen de afdeling BioMedical Engineering, het Chirurgisch Onderzoekslaboratorium en de kliniek heeft geresulteerd in een vruchtbare onderzoeksomgeving waarbij de toekomst met vertrouwen tegemoet kan worden gezien.

Verbeteren van statische koude preservatie

Dit proefschrift beschrijft een aantal studies dat er op gericht is orgaanpreservatie te verbeteren om op die manier de klinische resultaten na nier- en levertransplantatie te verbeteren. Het centrale dilemma in orgaanpreservatie is de disbalans die ontstaat tussen metabole vraag en aanbod die optreedt wanneer een orgaan uit de circulatie wordt genomen. De huidige (klinische) strategieën zijn erop gericht de metabole vraag te verminderen door het afkoelen van het orgaan tot 0-4°C. Dit resulteert in een afname van het metabolisme van ongeveer 90%. De bijkomende schade van hypothermie, celoedeem, acidose en zuurstofradicaal productie, zal echter altijd een rem blijven zetten op verdere ontwikkelingen in hypotherme preservatietechnieken. Nieuwe strategieën moeten zich focussen op een reductie in de metabole vraag van het orgaan óf een verbetering in metabole ondersteuning tijdens preservatie óf een combinatie van beide.

In plaats van hypothermie moeten andere mechanismen worden onderzocht om het metabolisme reversibel te verlagen. Dit is geen eenvoudige opdracht, maar de natuur kan ons een handje helpen. Orgaanpreservatie-onderzoekers zouden (winter)slapend rijk kunnen worden door de kunst af te kijken van bijvoorbeeld de eekhoorn. Dieren in winterslaap (hibernatie) zijn in staat lange perioden van extreem lage lichaamstemperatuur en bijbehorend laag metabolisme te overleven. Hibernatie is een conditie waarbij grote veranderingen in orgaanfunctie optreden. Een belangrijk aspect van deze processen is een significante reductie in zowel de energieproductie als -consumptie. Tijdens hibernatie zakt de lichaamstemperatuur naar omgevingstemperatuur (0-10°C), worden lichaamsfuncties en metabolisme tot een minimum beperkt en raken organismen in een lethargische staat. Dit kan enkele dagen tot weken duren. Gedurende deze periode zijn organismen beschermd tegen diverse omgevingsinvloeden zoals extreem lage temperaturen en zuurstofgebrek. Interessant is dat levers van eekhoorns, op het moment dat deze eekhoorns niet hiberneren (zomer), na 72 uur CS veel meer beschadigd zijn dan levers van dezelfde eekhoorns in hibernatie (winter). Dus, binnen dezelfde soort vindt er een transitie plaats van een minder tolerant (zomer) fenotype naar een super tolerant (winter) fenotype. Dit suggereert dat systemische en cellulaire mechanismen die leiden tot een toegenomen tolerantie voor ischemie/reperfusie, aan en uit kunnen worden gezet binnen één

individu. Het ophelderen van deze mechanismen kan grote implicaties hebben voor orgaanpreservatie. Een nieuwe stof waarvan inmiddels bekend is geworden dat het hibernatie kan induceren, in van origine niet-hibernerende soorten *(Szabó C, Nature Reviews 2007)*, is waterstofsulfide (H_2S). Dit kleurloze en wateroplosbare gas met de karakteristieke geur van rotte eieren is voornamelijk bekend geworden als het giftige gas dat uit moerassen omhoog borrelt. H_2S wordt echter ook in kleine hoeveelheden in mensen geproduceerd. Het behoort tot de biologische familie van mediatoren die gasotransmitters worden genoemd. Koolstofmonoxide (CO) en stikstofoxide (NO) behoren eveneens tot deze groep. H_2S heeft een breed scala aan biologische effecten die uiteindelijk leiden tot cytotoxische of cytoprotectieve effecten, afhankelijk van de dosering en het doelorgaan. De remmende werking op cytochroom-c-oxidase, een belangrijke stap in de ATP synthese, is waarschijnlijk verantwoordelijk voor de door H_2S geïnduceerde hibernatie. Als muizen worden blootgesteld aan lage concentraties van H_2S, vindt er een dosis afhankelijke reductie in lichaamstemperatuur en metabolisme plaats. De ademhalingsfrequentie daalt van 120 ademhalingen per minuut naar onder de 10 per minuut en de lichaamstemperatuur zakt tot 15°C. Op het moment dat de blootstelling aan H_2S eindigt, herstellen de dieren zonder meetbare restschade.

Al deze effecten van H_2S zouden kunnen bijdragen aan het verbeteren van (machinale) preservatie en moeten worden onderzocht. In het GMP systeem kan H_2S-gas via de oxygenator worden toegediend of middels het coaten van de slangen of orgaankamer met een H_2S-donor. Wanneer H_2S wordt toegepast tijdens statische preservatie technieken zou het coaten van de orgaanzak met een H_2S-donor een aantrekkelijke optie kunnen zijn. Het ligt voor de hand dat - naar analogie van de hibernerende eekhoorn - organen in hibernatie meer tolerant zijn voor ischemie/reperfusie schade. Veel vragen rondom de smalle therapeutische breedte van H_2S en de lange termijn effecten van blootstelling aan H_2S moeten worden onderzocht en beantwoord voordat klinische orgaanpreservatie gebruik kan gaan maken van de effecten van H_2S. Hopelijk is er tegen die tijd ook een oplossing gevonden om het transplantatieteam te beschermen tegen de stank van rotte eieren.

Toekomstige ontwikkelingen rondom het Groningse Machinale Perfusie (GMP) systeem

In dit proefschrift werden GMP prototypes ontwikkeld en onderzocht in een leverpreservatie- en een nier autotransplantatiemodel. Hoewel voor de nier kon worden aangetoond dat preservatie met het GMP systeem beter was dan statische koude preservatie, ontbreekt deze data voor de lever. In *Hoofdstuk VI* werd het systeem bovendien getest met onbeschadigde, perfecte donornieren van jonge varkens. Deze werden zonder warme ischemie en zonder kans op eventuele afstoting getransplanteerd. Toekomstige experimenten moeten ook preservatie en allo-transplantatie van DCD nieren en levers onderzoeken. Om de positie van het GMP systeem te bepalen moeten er vergelijkende studies met andere commercieel verkrijgbare (nier) HMP machines plaatsvinden. Hierdoor kan de relatieve bijdrage van de centrifugaal pompen en perfusaat oxygenatie, die uniek zijn voor het GMP systeem, worden bepaald.

Verschillende auteurs hebben beschreven dat veranderingen in de renale vaatweerstand (RVW) tijdens HMP voorspellend zouden kunnen zijn voor de nierfunctie na transplantatie. Alhoewel enige retrospectieve data beschikbaar zijn kunnen alleen de prospectieve data en monsters van de gerandomiseerde klinische studie *(Hoofdstuk VII)* deze hypothese staven. Door de gemeten RVW tijdens HMP te correleren met de klinische resultaten na niertransplantatie kan de waarde van RVW worden vastgesteld. Indien er een correlatie bestaat zal er een drempelwaarde kunnen worden berekend. De monsters van UW-MPS op diverse tijdspunten tijdens HMP kunnen worden gebruikt om nieuwe biomarkers te ontdekken. Deze kunnen dan, eventueel samen met de RVW, bijdragen aan een betere voorspelling van het transplantatieresultaat.

In plaats van het verlagen van de metabole vraag, zouden nieuwe technieken zich ook kunnen richten op het verbeteren van de metabole support tijdens preservatie. Wanneer tijdens preservatie de fysiologische omstandigheden van oxygenatie, beschikbaarheid van voedingsstoffen en het uitscheiden van metabolieten kunnen worden nagebootst zal de schade aan het orgaan zo klein mogelijk zijn. Het GMP systeem is op de Universiteit van Barcelona in Spanje al met succes gebruikt voor normotherme machinale perfusie (NMP) van varkenslevers. Als preservatievloeistof werd verdund donorbloed gebruikt. Vergeleken met CS, werd minder schade aan het lever parenchym en een betere synthetische functie na transplantatie gezien. Toepassing van NMP in de klinische situatie wordt momenteel beperkt door de volumineuze machinerie en de technische complexiteit. Toekomstige studies moeten zich richten op het verkleinen van de opstelling en op het gebruik van een andere preservatievloeistof. Want, zoals in *Hoofdstuk VIII* werd aangetoond, leidt verdund bloed tot endotheel activatie waardoor het wellicht niet de meest geschikte vloeistof is voor het normotherm machinaal preserveren van donororganen. Wellicht moeten nieuwe oplossingen voor normotherme preservatie synthetisch van aard zijn. Componenten van celkweekmedia kunnen dan zorgen voor metabole ondersteuning, colloïden moeten worden toegevoegd om oedeem te voorkomen en zuurstofdragers faciliteren de oxygenatie van het orgaan.

Terwijl alle studies in dit proefschrift zich richten op het verbeteren van de preservatie van het reeds uitgenomen donororgaan, begint orgaanpreservatie eigenlijk al eerder, namelijk in de donor. Het optimaliseren van de initiële uitwas tijdens de donor-operatie zal bijdrage aan de kwaliteit van de uitgenomen organen. In de huidige klinische praktijk wordt 6-14 liter van een preservatievloeistof bij 0-4°C met een hydrostatische druk van 75-100 cm H_2O in de donor geïnfundeerd om bloed uit te spoelen en de organen te koelen. Het toepassen van een gemodificeerd GMP systeem voor deze toepassing zal resulteren in een drukgestuurde, geoxygeneerde en continue perfusie van het gehele abdominale compartiment tijdens de donor-operatie (Fig. 1). In plaats van geïsoleerde perfusie van nier of lever kunnen alle buikorganen geperfundeerd worden door de aorta en vena cava te cannuleren. Door deze zogenaamde 'in-situ machine perfusie' zal de beschikbaarheid van zuurstof leiden tot meer metabole support, terwijl de continue perfusie zorgt voor een verbeterde uitwas van

Figuur 1. Prototype van het Extra Corporeal Organ Procurement System (ECOPS).

bloed en koeling van de organen. Het gecombineerde effect zal een reductie in de disbalans tussen metabole vraag en aanbod zijn. Als vervolgens de geïsoleerde orgaanpreservatie wordt gecontinueerd met HMP, ontstaat er een optimale en technisch haalbare preservatiemethode voor klinisch gebruik.

Abbreviations

αSMA	alfa-smooth muscle actin
AAP	alanine aminopeptidase
ACH	acetylcholine
ADP	adenosine diphosphate
AI	aggregation index
AMP	adenosine monophosphate
AQP	aquaporin
ATP	adenosine triphosphate
BPM	beats per minute
CEL	Celsior solution
CIT	cold ischemic time
CNI	calcineurin inhibitor
CO	carbon monoxide
CPB	cardio pulmonary bypass
CS	cold storage
CSS	cold storage solution
DBD	donation after brain death
DCD	donation after cardiac death
DGF	delayed graft function
EC	EuroCollins
ECD	expanded criteria donation
ED-1	ectodysplasin-1, infiltrating macrophage specific antibody
eNOS	endothelial nitric oxide synthase
f-DGF	functional DGF
FGS	focal glomerulosclerosis
GFR	glomerular filtration rate
GMP	Groningen Machine Perfusion
GS	graft survival
h	hour(s)
Hb	hemoglobine
Hct	hematocrit
HES	hydroxyethyl starch
HLA	human leucocyte antigen
HMP	hypothermic machine perfusion
HOC	hypertonic-citrate-solution (Marshall's solution)
HR	heart rate
HR	hazard ratio
HTK	Histidine-Tryptophan-Ketoglutarate
ICAM-1	intercellular adhesion molecule-1
IF	interstitial fibrosis
IGL-1	Institut Georges Lopez-1
IHD	isovolemic hemodilution

IPK	isolated perfused kidney
IL-6	interleukin-6
IL-18	interleukin-18
iNOS	inducible nitric oxide synthase
IRI	ischemia/reperfusion injury
IU	international units
IV	intravenous
kDa	kilo Dalton
LDH	lactate-dehydrogenase
LORCA	laser-assisted optical rotation cell analyzer
MAP	mean arterial pressure
MCP-1	monocyte chemotactic peptide-1
MDA	malondialdehyde
min	minute(s)
mmHg	millimeters mercury
MPS	machine perfusion solution
MW	molecular weight
NAG	N-acetyl-ß-D-glucosaminidase
NHB	non heart-beating
NHBD	non heart-beating donor
NMP	normothermic machine perfusion
NO	nitric oxide
NOS	nitric oxide synthase
OR	odds ratio
PBS	phosphate-buffered-sucrose solution
PE	phenylephrine
PEG	polyethylene glycol
PFC	perfluorocarbon
PNF	primary non function
PRA	panel-reactive antibody
PS	patient survival
RBC	red blood cell
RCT	randomized controlled trial
RVR	renal vascular resistance
ROS	reactive oxygen species
s	second(s)
SCD	standard criteria donation
SMCs	smooth muscle cells
TA	transplant arteriosclerosis
TBARS	thiobarbituric acid-reactive substances
TNFα	tumor necrosis factor-alfa
UW-CSS	University of Wisconsin cold storage solution
UW-MPS	University of Wisconsin machine perfusion solution
vWF	von Willebrand factor

WIT	warm ischemic time
yr	year(s)

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Mark-Hugo J. Maathuis was born in Apeldoorn, The Netherlands on March 4, 1979. He was educated at the 'Veluws College' Atheneum in Apeldoorn, and at the University of Groningen where he started to study medicine in 1997. He went abroad in 2001 for a seven-month period of clinical internships at the St. Elisabeth Hospital in Willemstad Curaçao, Dutch Antilles, and in 2003 for a four-month elective clinical rotation in transplant surgery at the University of Wisconsin Hospital and Clinics, Madison, U.S.A. In October 2003 he received his medical diploma cum laude and started in February 2004 as a research fellow at the Surgical Research Laboratory of the University Medical Center Groningen. In 2005 he became a European Society for Organ Transplantation (ESOT) trainee at the 'Haus für Experimentelle Therapie" of the University Clinic of Surgery of the Rheinische Friedrich Wilhelms Universität in Bonn, Germany. In 2007 he worked as a visiting research fellow at the Liver Transplant Unit of the Hospital Clinic Barcelona, Universitat de Barcelona, Spain. Mark-Hugo is living together with Jantien Kolkman and in the summer of 2008 their daughter Jiske was born.